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PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **Covacci *et al.***

Serial No.: **09/306,934** Group Art Unit: **1645**

Filed: **July 26, 1999** Examiner: **P. Bui**

For: **HELICOBACTER PYLORI CYTOTOXIN PROTEINS USEFUL FOR VACCINES AND DIAGNOSTICS**

I, **Robin S. Quartin**, Registration No. **45,028** certify that this correspondence is being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.
On Aug 22, 2000

Robin S. Quartin Reg. No. 45,028

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

I, Giuseppe Del Giudice, do hereby declare as follows:

1. I am a Research Director employed by Chiron SpA, in Siena, Italy.

2. I am a medical doctor (M.D.) with 15 years of experience in vaccine development.

3. I have read U.S. application serial number 09/306,934, filed July 26, 1999, and entitled "*Helicobacter pylori* cytotoxin proteins useful for vaccines and diagnostics" ("'934

application"). While I am not an inventor of the subject matter of the '934 application, I am quite familiar with the invention and the technology at issue. The '934 application claims priority to PCT/EP93/00472 (filed March 2, 1993) and PCT/EP93/00158 (filed January 25, 1993), which two PCT applications claimed priority benefit of Italian application Serial No. FI92A000052 (filed March 2, 1992).

4. The invention provides purified, recombinantly produced polypeptides of the *Helicobacter pylori* cytotoxin (VacA) protein[1], for use, among others, in vaccines. The component VacA polypeptides of such vaccines should induce an immune response, should not be toxic, and preferably, should protect against subsequent challenge by the pathogen *H. pylori*.

5. I have read the Official Action dated February 7, 2000 ("Action").

6. In the Action, the Examiner rejected claims 40 - 50 as allegedly being indefinite because of the use of the term "substantially," which the Examiner asserts is a relative term lacking comparative basis. I respectfully disagree.

7. The term "substantially" is used in the amended and original claims in conjunction with terms relating to toxicity in such phrases as "exhibits substantially no toxicity, or substantially reduced toxicity," and "exhibits no functional contribution to toxicity, or a substantially reduced functional contribution to toxicity." These phrases are used to describe the characteristics of the claimed *H. pylori* VacA polypeptides, and the *H. pylori* VacA polypeptide components of the claimed vaccines of the invention. The term "substantially," as it is used in the '934 application,

[1]In the specification of the '934 application, *H. pylori* cytotoxin is referred to as "CT." However, current terminology for the "vacuolating" cytotoxin protein of *H. pylori* is "VacA."

would have been, and is clearly understood by those of ordinary skill in the art, to mean that such *H. pylori* proteins, or fragments thereof, do not exhibit statistically significant cytotoxic effects and, thus, would be acceptable for use in human vaccines. Cytotoxicity can be routinely assessed in a variety of assays known to those of skill in the art, such as *in vitro* vacuolation of cells or cell lines, *e.g.* HeLa cells, and *in vivo* administration of purified VacA to mice to analyze gastric tissue damage.

8. In the Action, the Examiner rejected claims 40 - 50, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with the claims. The Examiner asserted that undue experimentation would have been required to determine which VacA (CT) polypeptides/proteins are effective for use as vaccines and which fragments of cytotoxin protein would "exhibit substantially no contribution to toxicity" or would be effective as components in a "prophylactic or therapeutic vaccine," at the time of filing. I respectfully disagree.

9. Chemically inactivated and genetically detoxified toxins were known to those of skill in the art, prior to March 2, 1992. For example, Exhibit A, which is a copy of Manetti *et al.*, 1995, *Infect. Immun.* 63:4476-4480, cites to prior studies (Pizza *et al.*, 1989) of chemically and genetically detoxified subunits of pertussis toxin, which also were capable of inducing protective immunity.

10. It is routine to determine, and was routine to determine, as of March 2, 1992, which polypeptide fragments of *H. pylori* VacA would exhibit substantially no toxicity, or substantially reduced toxicity, be immunogenic, and be functional as a vaccine.

11. Protein fragments for testing are easily generated through such means as recombinant expression techniques, using the sequences disclosed in the '934 application. Thus, it would have been routine, as of March 2, 1992, to generate fragments of *H. pylori* VacA protein and fragments of cytotoxin associated immunodominant antigen (CagA)[2]. Such fragments could then be used for further determinations of toxicity, immunogenicity, and vaccine efficacy.

12. Regarding toxicity, *in vitro* vacuolation assays can be used for the routine determination of toxic and non-toxic VacA protein, or fragments thereof. Exhibit B is a copy of Reyrat *et al.*, 1999, *Mol. Microbiol* 34:197-204, which is a review of the structure and activity of VacA. VacA was first described to induce vacuolation (the formation of large vacuoles) of mammalian cells *in vitro*, in 1988 (*see* page 199, column 2 of Exhibit B). Fig. 1A of Exhibit B shows a microscopic view of the vacuolating activity of purified VacA protein on HeLa cells. Thus, at the time of filing, those skilled in the art could have used an *in vitro* vacuolation assay on mammalian cells to routinely distinguish toxic and non-toxic VacA fragments.

13. Animal models used for the study of *H. pylori* infection were known prior to March 2, 1992. Such animal models of *H. pylori* infection include the gnotobiotic piglet (Krakowka *et al.*, 1987, Infect. Immun. 55:2789-2796) (Exhibit C) and the gnotobiotic dog (Radin *et al.*, 1990, Infect. Immun. 58:2606-2612) (Exhibit D). Animal models present convenient *in vivo* assay systems for routinely distinguishing non-toxic VacA protein, or fragments thereof, from those which exhibit toxicity. For example, Telford *et al.*, 1994, *J. Exp. Med.* 179:1653-1658 (Exhibit E), describes an *in vivo* mouse model of *H. pylori*-induced gastric ulceration, used to show that the VacA protein is responsible for the epithelial erosion seen in *H. pylori* infection. Sonicated, VacA-producing *H.*

[2]In the specification of the '934 application, *H. pylori* cytotoxin associated immunodominant antigen is referred to as "CAI." However, the current terminology used for this protein is the "cytotoxin-associated gene A" or "CagA" antigen.

pylori cells were shown to induce erosive lesions of the gastric mucosa, when orally administered to mice (Figure 1 (b and c)). Administration of purified VacA to mice also resulted in extensive tissue damage and mucosal erosion (Figure 1 (d, e, and f)). Thus, *H. pylori* infection models can be used, and could have been used at the time of filing, for routinely distinguishing toxic and non-toxic VacA fragments.

14. Regarding immunogenicity, one of skill in the art could have employed classical immunological assays to screen for antibody production in response to immunizations with fragments of *H. pylori* cytotoxin protein. These include, for example, 1) enzyme-linked immunosorbent assay (ELISA), 2) proliferation assays of cells from lymphoid organs, and 3) evaluation of the number of cells producing antibodies to a given antigen. Detailed protocols for these standard assays can be found in any manual on immunology. The Handbook of Experimental Immunology, Weir & Blackwell (eds.), 1986, which is cited at page 5, lines 22 - 24 of the specification, is a good example of such a manual, available to those of skill in the art at the time of filing of the application. Current Protocols in Immunology, John Wiley & Sons, New York, NY, which has been published since 1991, is another example of such a manual available to those of skill in the art. Thus, it would have been routine to determine which fragments of cytotoxin protein would generate an immune response, at the time of filing of the '934 application.

15. Regarding vaccine function, at page 15, lines 14 - 17, the specification of the '934 application defines a vaccine as "capable of eliciting protection against *H. pylori*." Furthermore, the vaccines of the invention can be prophylactic, therapeutic, or both (*see* page 38, line 39 - page 40, line 2, of the '934 application). Demonstration of a prophylactic or therapeutic effect of a protein, or polypeptide fragment of a protein, could have been carried out using routine functional experiments and assays. Functional experiments include the administration of a candidate vaccine to animals susceptible to *H. pylori* infection, either before challenge with the pathogen (prophylaxis

determination) or after infection has taken place (treatment determination). Animal models of disease provide convenient environments for such vaccine testing. *See* ¶ 13 above.

16. Exhibit F is a copy of Nedrud, 1999, *FEMS Immunol. Med. Microbiol.* 24:243-50, which is a review of animal models of *H. pylori* infection that have been established, including the pig, dog, gerbil, monkey, and ferret. Such models have been used since 1987 to examine infection-related disease processes and evaluate vaccines, and they can be used routinely to determine the effectiveness of *H. pylori* proteins and polypeptide fragments as vaccines against the infection.

17. Exhibit G is a copy of Ghiara *et al.*, 1997, *Infect. Immun.* 65:4996-5002, of which I am a co-author, and which shows that therapeutic vaccination with full-length recombinant *H. pylori* proteins, including VacA, can eradicate chronic *H. pylori* infection in a mouse model, and protect against subsequent challenge. Figure 3 of Exhibit G presents the results of therapeutic vaccination. Vaccination with full-length recombinant VacA protein (indicated as "Tox100") resolved the infection in about 92% of the mice. Full-length recombinant CagA protein yielded a 70% eradication of infection rate. Furthermore, once therapeutically treated, the mice are also protected from further challenge with *H. pylori*. Figure 5 presents the results of a study of reinfection rate, and shows that therapeutic vaccination with the recombinant VacA protein protected 70% of the mice from reinfection with *H. pylori*.

18. In the Action, the Examiner also asserted that a mucosal adjuvant is required for effective *H. pylori* component vaccines. I respectfully disagree with this characterization of the state of the art. Exhibit H is a copy of published PCT application PCT/IB99/00851, of which I am a co-inventor, and which teaches that mucosal delivery and mucosal adjuvants are <u>not</u> required for effective *H. pylori* component vaccines. This PCT application presents the results of intramuscular immunization studies with *H. pylori* component vaccines, in a dog model. Page 21 presents the

protocol for immunization. Full-length recombinant *H. pylori* proteins -- VacA, CagA, and neutrophil activating protein (NAP) -- were used as vaccine components for intramuscular immunizations. The adjuvant was aluminum hydroxide, *i.e.*, not a mucosal adjuvant.

19. Exhibit H demonstrates that the intramuscular immunizations induced high serum titers of antigen-specific antibodies to each of the *H. pylori* component proteins in the vaccine (*see* Figure 5A (VacA), Figure 5B (CagA), and Figure 5C (NAP)). Furthermore, intramuscular immunization was effective to protect all of the dogs from challenge with *H. pylori*. No symptoms of *H. pylori* infection were evident in the intramuscularly vaccinated dogs (page 12, lines 24 - 25). At 10 and at 42 days post-challenge, the intramuscularly vaccinated dogs' antral biopsies and gastric lavages were negative for urease[3] activity (page 12, lines 26 - 29, and page 14, Table 2). Furthermore, at 42 days post-challenge, intramuscularly vaccinated dogs had normal mucosa, without the signs of hyperemia or edema seen in the *H. pylori*-infected control animals (page 14, lines 4 - 10, and Figures 1A and 1B).

20. In the Action, the Examiner also rejected the claims as anticipated by Cover *et al.* (1992). I respectfully disagree. Cover *et al.* (1992) describes purified, native *H. pylori* 87kDa cytotoxin protein and reports that it is toxic. Cover *et al.* (1992) also describes the immunization of a rabbit with SDS-PAGE purified, denatured cytotoxin to generate an antisera, containing antibodies specific for the 87 kDa cytotoxin protein (as shown by immunoblot), and capable of neutralizing native cytotoxin vacuolating activity.

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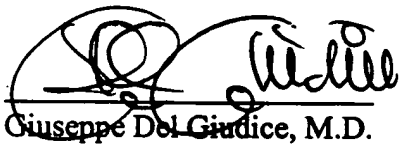
[3]Urease is another *H. pylori* protein (*see* '934 application page 2, lines 8 - 14). A urease activity assay (*see* '934 application page 46, lines 14 - 16) is a means of detecting the presence of *H. pylori* infection, both in the experimental and the clinical setting.

21. The claims of the '934 application are directed to (1) recombinantly produced *H. pylori* VacA polypeptides, exhibiting substantially no toxicity, or substantially reduced toxicity, (2) vaccines comprising VacA polypeptides of (1), exhibiting substantially no toxicity, or substantially reduced toxicity, and (3) methods of making these vaccines. Manetti *et al.* (1995) (Exhibit A) shows a recombinant VacA protein that is non-toxic. The results show that the recombinant VacA protein, and two recombinant fragments of VacA protein, are non-toxic, having no vacuolating activity *in vitro* (page 4478, column 1). Although Manetti *et al.* (1995) (Exhibit A) reports that antibodies made against the denatured recombinant protein were unable to block the *in vitro* vacuolating activity of purified, native VacA (page 4478, Figure 4), Ghiara *et al.* (1997) (Exhibit G) disclose that the recombinant VacA is, nevertheless, capable of generating a protective effect against *H. pylori* infection.

22. Cover *et al.* (1992) does not disclose a recombinantly produced *H. pylori* VacA polypeptide exhibiting substantially no toxicity, or substantially reduced toxicity.

23. I declare that all statements made herein are of my own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Giuseppe Del Giudice, M.D.

17 August 2000
Date

EXHIBIT A

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Helicobacter pylori Cytotoxin: Importance of Native Conformation for Induction of Neutralizing Antibodies

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We have attempted to express the *Helicobacter pylori* vacuolating cytotoxin in *Escherichia coli*. Although the 95-kDa VacA polypeptide was expressed abundantly, it completely lacked any biological activity. In addition, this material failed to induce neutralizing antibodies after immunization of rabbits. In contrast, highly purified high-molecular-mass cytotoxin from the supernatant of *H. pylori* cultures was active in a HeLa cell assay and effectively induced a neutralizing response in rabbits. Neutralizing sera were shown to contain a high proportion of antibodies which recognized conformational epitopes found only on the native toxin. The data indicate that toxin-neutralizing epitopes are conformational and that potential vaccines based on the cytotoxin may benefit from the use of the intact molecule.

Helicobacter pylori infection is now recognized as the cause of most gastroduodenal disease, including chronic active gastritis, atrophic gastritis, and peptic ulcer (1). Recent data indicate that a subgroup of *H. pylori*, referred to as type I strains (17), which produce a potent cell-vacuolating cytotoxin (5) may be responsible for the more severe forms of disease (2, 15). In support of this hypothesis, extracts of cytotoxic (type I) strains but not noncytotoxic (type II) strains cause epithelial damage and ulceration when administered orally to mice. Furthermore, the highly purified toxin causes lesions similar to those observed after administration of whole-cell extracts (15). More recently, in a mouse model of *H. pylori* infection, cytotoxic strains but not noncytotoxic strains caused gastric lesions similar to those seen in severe disease in humans (6). Hence, study of the cytotoxin will be important to our understanding of *H. pylori*-induced disease, and the cytotoxin may be an important candidate for therapeutic or prophylactic vaccines.

The toxin is synthesized in *H. pylori* as a 140-kDa precursor protein with a 33-amino-acid amino-terminal signal peptide, which is presumably responsible for secretion across the plasma membrane (16). The carboxy-terminal 45-kDa segment of the precursor is structurally similar to the outer membrane transporter region of the immunoglobulin A protease family of exotoxins (13). This region is cleaved from the molecule during export across the outer membrane and remains associated with the bacterial cell (16). The 95-kDa mature portion of the toxin is released slowly from the bacteria and is found in the culture medium as a high-molecular-mass oligomer (3). The 95-kDa monomer is further processed in the culture medium by specific cleavage to produce an amino-terminal subunit with a size of 37 kDa and a carboxy-terminal subunit with a size of 58 kDa (16). The subunits copurify on gel filtration with the intact molecule, indicating that they remain associated after cleavage. The physiological relevance of this further processing is not yet clear, but it may be important for activation of the toxin, as is the case for several other bacterial toxins.

Large-scale production of the toxin is essential for studies of function and for assessment of its potential as a vaccine. For this reason, we have expressed the 95-kDa mature portion of the toxin and each of the two subunits separately in *Escherichia coli* and compared these products with highly purified native toxin in activity and ability to induce neutralizing antibodies in rabbits. The antisera raised against the recombinant products recognized the native toxin in enzyme-linked immunosorbent assay (ELISA) but failed to neutralize the toxic activity. Highly purified native toxin could be produced in milligram quantities and was highly potent in inducing neutralizing sera.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* M15 (pRep4) (Qiagen Inc., Chatsworth, Calif.) was used for cloning experiments. Recombinant bacteria were grown in Luria-Bertani broth or agar plates supplemented with ampicillin (100 μg/ml) and kanamycin (25 μg/ml). *H. pylori* reference strain NCTC 11637 and strain 60190 (kindly supplied by M. J. Blaser) were grown on Columbia agar with 5% horse blood, 0.2% cyclodextrin, and Dent or Skirrows antibiotic supplement (Oxoid, Basingstoke, United Kingdom). Liquid cultures were grown in 5-liter bioreactors in brucella broth supplemented with 2% cyclodextrin (7).

Expression of recombinant toxin. DNA cloning procedures were carried out according to standard protocols (12). *H. pylori* DNA fragments were generated either by restriction enzyme digestion of the cloned gene (16) or by PCR amplification with specific primers. pTOX140 was constructed in two steps as follows. (i) An approximately 5-kbp fragment from the *Eco*RI site at position 491 of the published sequence (16) to a *Bam*HI site approximately 1.5 kbp after the stop codon was cloned into the respective sites in pQE30 (Qiagen). This construct thus lacked the sequences coding for the ribosome binding site, initiator methionine, and histidine tag of the pQE30 plasmid. (ii) A short fragment synthesized by PCR from a synthetic *Eco*RI site at position 201 of the published sequence to the *Eco*RI site at position 491 was inserted into the plasmid from step i. Thus, the construct contains the pQE30 promoter followed by the ribosome binding site and the entire gene encoding the cytotoxin. A similar strategy was used for the construction of pTOX100 except that a *Bam*HI site was added immediately preceding base 316 of the published sequence and the insert terminated at the *Hin*dIII site at position 3215 of the published sequence. Thus, the region coding for amino acids 34 to 1000 was cloned in frame with the sequences coding for the histidine tag in the *Bam*HI-*Hin*dIII sites of pQE30. pTOX37 and pTOX58 were constructed by cloning the sequences coding for amino acids 34 to 352 and 353 to 995 in the *Bam*HI-*Sal*I sites of pQE30 with synthetic oligonucleotides with the appropriate restriction enzyme recognition sites.

Recombinant protein was purified on Ni-nitrilotriacetic acid resin (Qiagen), according to the manufacturer's instructions. Recombinant protein was bound to the column in 8 M urea–0.1 M sodium phosphate–0.01 M Tris (pH 8.0) and eluted with 6 M guanidinium hydrochloride–0.2 M acetic acid. Protein refolding was carried out by dialysis against 50 mM β-alanine (Sigma Chemical Co., St. Louis, Mo.) (pH 3.8)–10% glycerol–5 mM glutathione (reduced)–0.5 mM glu-

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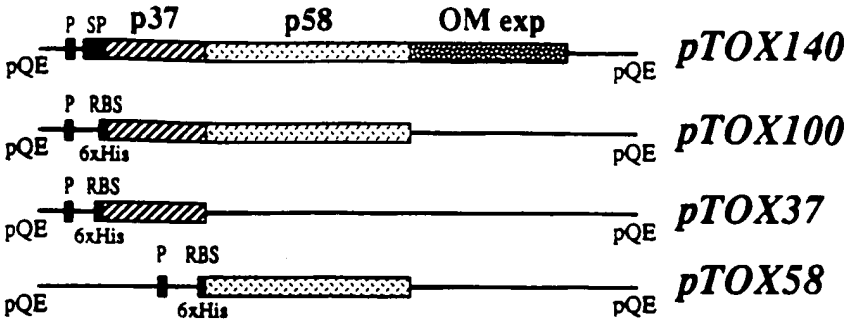

FIG. 1. Schematic representation of plasmid constructs used for expression of cytotoxin in *E. coli*. P, promoter; RBS, ribosome binding site; SP, signal peptide; OM exp, putative outer membrane exporter; 6xHis, tag of six histidine residues.

tathione (oxidized) and then dialysis stepwise against 50 mM β-alanine–10% glycerol at pH 4.5, 5.5, and 7.0. Rabbits were immunized intradermally with five doses, spaced 7 days apart, of 100 μg of recombinant proteins in Freund's adjuvant. Immunoblotting was carried out by using a chemiluminescence detection kit (ECL; Amersham) and horseradish peroxidase-labeled anti-rabbit immunoglobulin antibodies.

Purification of native VacA. The biomass from a nominal 5-liter culture of *H. pylori* CCUG was removed by centrifugation at 11,000 × *g* for 20 min, and the supernatant liquid (approximately 4 liters) was brought to 50% saturation by the addition of solid ammonium sulfate. The suspension was centrifuged at 11,000 × *g* for 20 min, and the precipitated proteins were resuspended in 100 mM NaCl–20 mM phosphate (pH 6.5) and dialyzed extensively against the same buffer. The dialysate was adjusted to a volume of 250 ml and applied, at a flow rate of 2.5 ml/min, to a column (2.5 by 11 cm, Econo column; Bio-Rad, Hercules, Calif.) containing Matrex Cellufine Sulfate (Amicon, Danver, Mass.). The proteins were washed extensively with loading buffer, eluted from the column with a gradient of 0.1 to 1.5 M NaCl in 20 mM phosphate buffer (pH 6.5), and monitored by A_{280}. The eluate between 0.5 and 0.8 M NaCl containing the VacA protein was collected and brought to 50% saturation with ammonium sulfate. The suspension was centrifuged at 11,000 × *g* for 20 min, and the pelleted proteins were resuspended in phosphate-buffered saline (PBS) (300 mM NaCl). Insoluble material was removed by centrifugation at 25,000 × *g* for 5 min, and the cleared solution was applied to a column (16 by 81 mm) packed with Superose 6 (Pharmacia, Uppsala, Sweden). Proteins were eluted with PBS and monitored by A_{280}. Fractions containing highly purified VacA, as assessed by Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were pooled. The protein concentration in the purified fractions was measured by using the Dc protein assay reagent kit (Bio-Rad) with bovine serum albumin as a standard.

Immunization of rabbits and neutralization of toxin. New Zealand White rabbits approximately 2.5 kg in size were immunized by intradermal injections of 25 μg each of purified toxin in a solution of 1 mg of aluminium hydroxide per ml as the adjuvant on days 0, 14, 28, and 56. Immunoglobulins were purified by using protein G-Sepharose (Pharmacia) and tested for their ability to neutralize VacA cytotoxic activity in an in vitro vacuolation assay (9) in which serial dilutions of the purified immunoglobulins were incubated with 1.85 μg of purified toxin in 40 μl of PBS added to the cells in 160 μl of culture medium containing 5 mM ammonium chloride.

ELISA. Flat-bottomed 96-well plates were coated by incubation overnight at 4°C with native VacA (2 μg/ml) or recombinant TOX100 (10 μg/ml) in PBS. After thorough washing with PBS–0.05% Tween 20, the plates were blocked by incubation for 2 h at 37°C with a solution of polyvinylpyrrolidone 360000 (2.7%) (Sigma Chemical Co.) and then washed again. Serial dilutions of purified immunoglobulins from anti-VacA and anti-TOX100 rabbit antisera in PBS–0.05% Tween 20 were incubated in the coated plates for 1.5 h at 37°C. The plates were washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antibodies for 1.5 h at 37°C. After the plates were washed, the reaction was developed by using the ELISA *In vitro* Test (Sclavo Diagnostics, Siena, Italy).

RESULTS

Expression of vacuolating cytotoxin in *E. coli*. Plasmid pTox140 contains the entire *H. pylori* toxin gene including sequences coding for the signal peptide and putative outer membrane exporter cloned downstream of a synthetic inducible promoter in the plasmid vector pQE30 (shown schematically in Fig. 1). This construct was introduced into *E. coli* M15, and the expression of the toxin gene was induced by treatment with IPTG (isopropyl-β-D-thiogalactopyranoside). Despite the fact that the synthetic promoter in pQE30 is very strong, only trace quantities of a 140-kDa protein which reacted in Western blots (immunoblots) with antitoxin antisera could be detected in the cell extracts (Fig. 2A). No processed 95-kDa protein could be detected either in the cell extracts or in the culture supernatant. *E. coli* would thus appear to be incapable of correct synthesis, processing, and export of the full-length cytotoxin precursor.

To overcome this problem, we attempted to express the region of the gene coding only for the mature 95-kDa portion of the protein either intact or in two pieces corresponding to the 37- and 58-kDa subunits. To facilitate purification of these proteins, they were expressed with six amino-terminal histidines, which enable purification by nickel chelation chromatography. The plasmid constructs used are shown schematically in Fig. 1. Each of these proteins was expressed to high levels after induction in *E. coli* (Fig. 2B); however, the proteins precipitated inside the bacteria and were found in the insoluble fraction of the cells after rupture by sonication.

We attempted to solubilize and renature the recombinant proteins to produce functional toxin. The proteins were solubilized in 8 M urea, purified on nickel-chelating columns, and

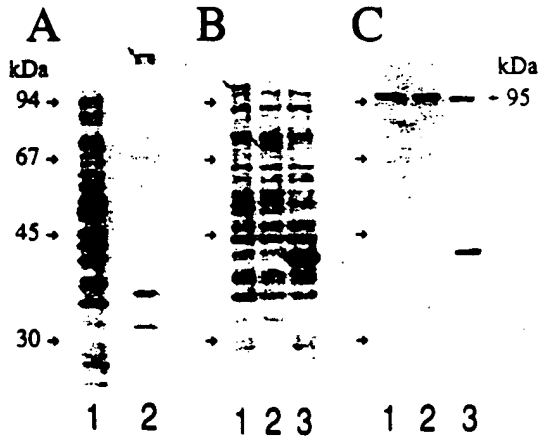

FIG. 2. Expression of cytotoxin polypeptides in *E. coli*. (A) Coomassie blue staining (lane 1) and immunoblot with anticytotoxin antibodies (lane 2) of TOX140 in *E. coli* extracts after SDS-PAGE. The angled arrow shows the position of the 140-kDa precursor. (B) Coomassie blue staining of TOX100 (lane 1), TOX58 (lane 2), and TOX37 (lane 3) in extracts of *E. coli* expressing the respective plasmids. The angled arrows indicate the recombinant proteins. (C) Immunoblot of extracts of *H. pylori* NCTC 11637 with rabbit antisera raised against TOX100 (lane 1), TOX58 (lane 2), and TOX37 (lane 3). The positions of protein molecular mass standards are indicated (horizontal arrows).

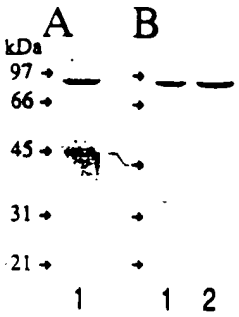

FIG. 3. SDS-PAGE and immunoblot of purified cytotoxin. (A) Coomassie blue staining of purified cytotoxin after SDS-PAGE; (B) immunoblot of the material in panel A (lane 2) and a similar preparation (lane 1) with antisera raised against TOX100. The migration of protein molecular mass standards is shown.

dialyzed stepwise to remove the urea. Although the proteins treated in this way remained in solution, no activity could be detected in a HeLa cell vacuolation assay (9). Rabbit antiserum raised against this material reacted well in a Western blot with the 95-kDa toxin protein in *H. pylori* extracts (Fig. 2C) and recognized the native protein at a high dilution in ELISA (see below); however, none of the serum samples was capable of neutralizing toxin activity in the in vitro vacuolation assay (data not shown).

Purification of native cytotoxin. The above results indicated that protective epitopes in the toxin molecule may be conformational and that the *E. coli*-expressed toxin was incapable of folding correctly. To test this and to establish whether the cytotoxin could be considered as a possible vaccine candidate, we needed to purify sufficient native active toxin to immunize rabbits. Published procedures for the purification of the cytotoxin have proven difficult for the preparation of large quantities. To overcome this problem, we developed a new, simplified procedure which gives reproducibly good yields of active cytotoxin. Supernatants from *H. pylori* NCTC 11637, cultured in bioreactors under conditions which ensure a high level of cytotoxin synthesis (7), were first concentrated, and then the toxin was purified by using a two-step procedure involving affinity chromatography and gel filtration (see Materials and Methods). The active cytotoxin eluted from the gel filtration in fractions corresponding to an apparent molecular mass of around 700 kDa. Coomassie blue staining of this material after SDS-PAGE revealed a single polypeptide with a molecular mass of 95 kDa which reacted with antiserum raised against the recombinant cytotoxin (Fig. 3). In some preparations, traces of the previously described 37- and 58-kDa processed products of the 95-kDa monomer (16) could be detected (Fig. 3B, lane 1).

With this procedure, milligram quantities of active cytotoxin were obtained from the culture supernatant. The estimated molecular mass of the toxin, although slightly lower, is within the limits of the technique which gave the value of 900 kDa previously reported (3).

Induction of neutralizing antisera by native cytotoxin. The purified native toxin proved very efficient at inducing neutralizing antisera in rabbits. Figure 4 shows the dose response of neutralization of purified toxin in a HeLa cell vacuolation assay. Antisera from two different rabbits gave similar results of 50% inhibition at around 10 μg of purified immunoglobulins per ml. Hence, it appears that conformati nal epitopes important for neutralization and thus for toxic activity are formed only on assembly of the high-molecular-mass oligomeric structure of the native toxin.

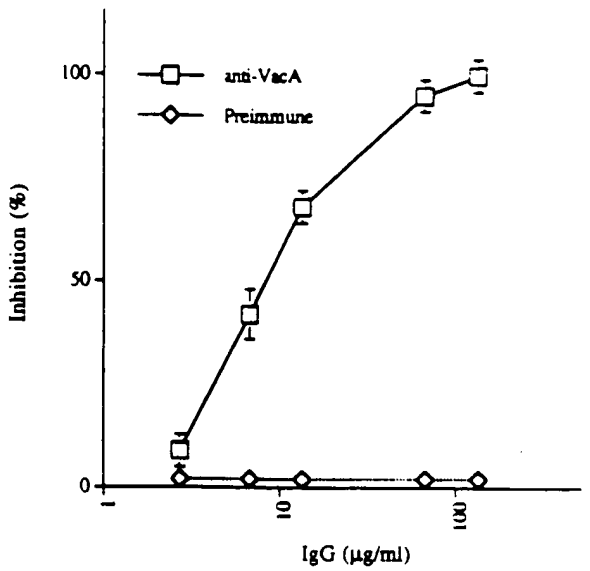

FIG. 4. Inhibition of cytotoxic activity by antibodies raised against purified native cytotoxin. Solid line, protein G-Sepharose-purified immunoglobulins from anticytotoxin serum; broken line, purified immunoglobulins from preimmune serum. The figure shows the mean and standard deviation of triplicate samples from representative experiments.

H. pylori isolates show a large degree of genetic variability. Nevertheless, cytotoxic activity extracted from an independent strain, *H. pylori* 60190 (3), isolated in a different geographic location was neutralized by similar concentrations of the rabbit antisera (data not shown). Even though the primary sequence of the toxin from this strain is only 93% identical to that of the protein used for immunization (4, 16), the protective epitopes are sufficiently conserved to allow effective cross-reaction.

Conformational epitopes in native toxin detected by ELISA. Figure 5 shows a comparison of antibody titers between rabbit antisera raised against native and recombinant cytotoxins. While sera raised against the different antigens had similar titers in ELISAs against the recombinant protein, they differed greatly when measured against the native protein. Antisera raised against the native toxin reacted with the native toxin at a considerably higher dilution than with the recombinant protein, indicating that a large part of the antibodies recognized conformational epitopes not present in the denatured molecule. In contrast, much higher concentrations of the antisera against the recombinant protein were required to react with the native protein, probably because some linear epitopes present in the denatured protein were masked in the correctly folded structure.

DISCUSSION

We have shown that native oligomeric toxin purified from cultures of *H. pylori* is capable of inducing high titers of neutralizing antibodies in rabbits whereas recombinant toxin proteins expressed in *E. coli* are not. The *E. coli*-expressed proteins were inactive in a HeLa cell assay of vacuolation, suggesting that the r ason that they failed t induce a neutralizing response was because they failed to fold correctly. Hence, the neutralizing epitopes in the native toxin protein are likely to be conformati nal and may be formed only when the protein folds correctly into its native structure.

This interpretation is supported by the results of the ELISA

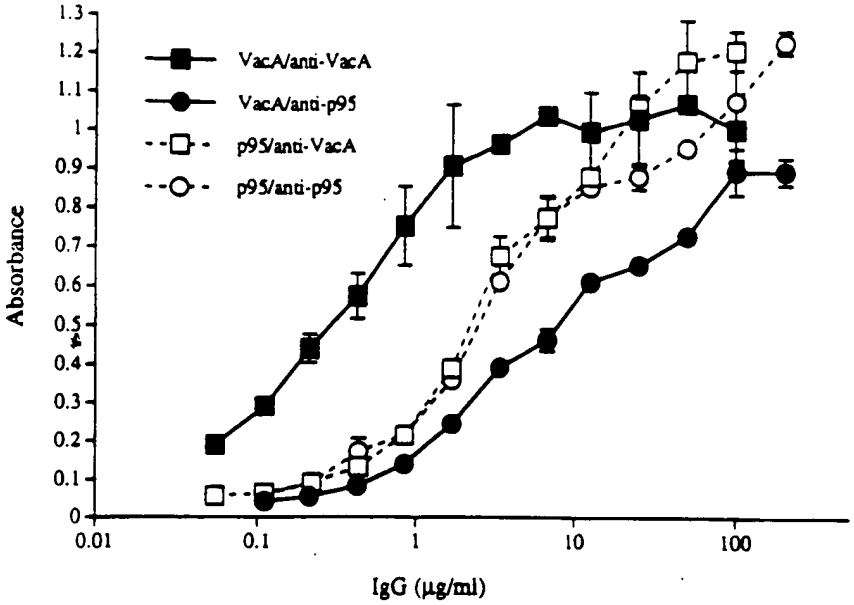

FIG. 5. ELISA determination of antibody titer in neutralizing rabbit antisera raised against native cytotoxin (squares) or recombinant TOX100 (circles) against the native antigen (solid lines, filled symbols) or the recombinant antigen (broken lines, open symbols).

using the native and recombinant proteins. Since both the responses of individual rabbits and the efficiency of binding of the antigens to the plate are likely to vary, the interpretation of these data relies on the crosswise assay of the titer of each antiserum with both the native and recombinant antigens. Antisera raised against the native antigen recognized the native protein at titers much higher than those of the recombinant protein. This difference in titers was not due to large differences in the quantities of antigens in the assay, as demonstrated by the fact that the antisera against the recombinant antigen reacted with the recombinant protein at a higher titer than with the native protein. The differential response of the two serum samples indicates that the native molecule is highly structured such that the immune response is due primarily to conformational epitopes. These data may be highly relevant to studies of serum antibodies against the toxin in *H. pylori*-infected individuals. It is likely that use of the recombinant protein will seriously underestimate the antibody titers and that only the native toxin will give accurate measurements of the host response during infection.

The native toxin has been reported to migrate in gel filtration with an apparent molecular mass of >900 kDa (3). Our estimate of the mass of the purified toxin is closer to 700 kDa, but this difference is within the limits of error of the preparative gel filtration used. Since only the purified protein was active and capable of inducing a neutralizing response, it is likely that the high-molecular-mass form of the toxin is not simply a nonspecific aggregate of toxin monomers but that it is an ordered structure required for activity. In support of this interpretation, visualization of the native toxin by electron microscopy has revealed a regular heptameric structure (unpublished data).

The data and conclusions are similar to those reported for the pertussis toxin, which is a heteropentameric protein. Antisera raised against the individual subunits failed to neutralize the toxin (8) whereas chemically inactivated or genetically detoxified holotoxins (10) are effectiv at inducing protective immunity (11). The pentameric structure of pertussis toxin has subsequently been characterized, and pertussis toxin, like several other multimeric bacterial toxins, has been shown to possess functional regions which span several subunits (14). The oligomeric structure of the *H. pylori* cytotoxin is likely to have similar properties.

The integrity of the native conformation of a vaccine can be extremely important for the induction of protective immunity. Even partial destruction of the conformational epitopes by chemical inactivation can result in lowering the effective immunogenicity. This variation in effectiveness has been amply demonstrated by inactivation of the pertussis toxin. Pertussis toxin genetically detoxified by substitution of two key amino acids in the enzymatically active site such that the structure is virtually unaltered has proven to be a considerably more effective immunogen than chemically inactivated forms (11). This possibility will have to be taken into consideration in the assessment of the cytotoxin as a possible vaccine candidate. A genetically detoxified molecule which retains the native structure will be an important goal.

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EXHIBIT B

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MicroReview

Towards deciphering the *Helicobacter pylori* cytotoxin

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Summary

VacA, the major exotoxin produced by *Helicobacter pylori*, is composed of identical 87 kDa monomers that assemble into flower-shaped oligomers. The monomers can be proteolytically cleaved into two moieties, one of 37 and the other of 58 kDa, named P37 and P58 respectively. The most studied property of VacA is the alteration of intracellular vesicular trafficking in eukaryotic cells leading to the formation of large vacuoles containing markers of late endosomes and lysosomes. However, VacA also causes a reduction in transepithelial electrical resistance in polarized monolayers and forms ion channels in lipid bilayers. The ability to induce vacuoles is localized mostly but not entirely in P37, whereas P58 is mostly involved in cell targeting. Until recently, *H. pylori* isolates were classified as tox$^+$ or tox$^-$, depending on whether they induced vacuoles in HeLa cells or not. Today, we know that almost all strains are cytotoxic. The major difference between tox$^+$ and tox$^-$ resides in the cell binding domain, which exists in two allelic forms, only one of which is toxic for HeLa cells. The two forms, named m1 and m2, are found predominantly in Western and Chinese isolates respectively.

Historical overview

Helicobacter pylori is a Gram-negative bacterium identified some 15 years ago as the causative agent of chronic gastritis and peptic ulcer (Warren and Marshall, 1983).

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Contrary to most bacterial infectious diseases, *H. pylori* gives a long-lasting infection with a time-scale in years or even decades (Blaser, 1993). Furthermore, *H. pylori* was classified as a type I carcinogen as it favours the development of gastroduodenal carcinoma and gastric lymphoma (Parsonnet *et al.*, 1991, 1994).

Soon after the isolation of *H. pylori*, it was discovered that broth culture filtrates of the majority of clinical isolates exhibited a cytotoxic activity (Fig. 1A) characterized by the vacuolation of diverse mammalian cell lines and the induction of gastric epithelial erosion in animal models (Leunk *et al.*, 1988; Kamiya *et al.*, 1994; Telford *et al.*, 1994; Marchetti *et al.*, 1995; Cover, 1996). Purified to homogeneity, the agent responsible for this toxic activity proved to be a heat-resistant oligomeric protein composed of identical 87 kDa monomers (Cover and Blaser, 1992; Cover, 1996; Lupetti *et al.*, 1996; Yahiro *et al.*, 1997). Vacuolating activity is strongly potentiated by a short exposure to acidic pH, a treatment leading to a profound molecular reorganization of VacA as assessed by circular dichroism and fluorescence studies (de Bernard *et al.*, 1995; Molinari *et al.*, 1998a). Amino-terminal sequencing of the first 23 amino acids greatly facilitated subsequent genetic characterization of VacA. Four groups reported the sequence of the *vacA* gene (Cover, 1996), which is about 4 kb in size (Fig. 1B) with an orthodox N-terminal signal sequence and a C-terminal domain which is cleaved during export across the outer membrane (Schmitt and Haas, 1994; Wang *et al.*, 1998a). Interestingly, this C-terminal domain belongs to a family of autotransporters widely used for the export of virulence factors in Gram-negative bacteria (Loveless and Saier, 1997) such as AidA (adhesin) in *Escherichia coli*, IgAP (IgA protease) in *Neisseria gonorrhoeae* and Ssp (serine protease) in *Serratia marcescens*.

VacA is a flower

The 87 kDa monomer of VacA can be cleaved into two subunits, one of approximately 37 and the other of 58 kDa (Telford *et al.*, 1994), named P37 and P58 respectively, following specific proteolysis of a flexible surface-exposed loop (Fig. 1B). However, it is still unknown whether this cleavage activity is due to VacA itself or to

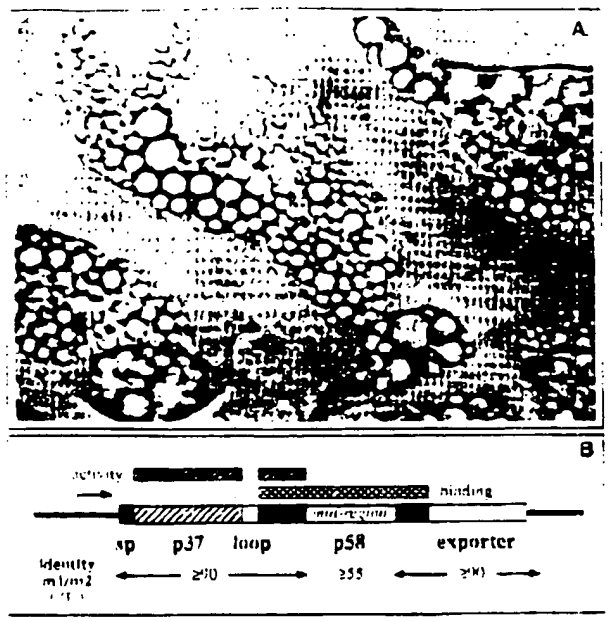

Fig. 1. A. Phase-contrast microscopy of HeLa cells vacuolated by 5 μg.ml^{-1} of purified VacA.
B. Schematic representation of the wild-type *vacA* gene and the product obtained. The level of amino acid identity between m1 and m2 forms is indicated below. sp, signal peptide; loop, flexible loop between P37 and P58 containing the proteolytic cleavage site. Above the schematic representation of the *vacA* gene, the minimal intracellular vacuolating domain and the minimal cell binding domain are represented by black and grey hatched boxes respectively.

a surface-associated protease. It is not yet known whether processing into two subunits is required for toxic activity. The molecular weight of the native toxin was found to be > 600 kDa using a gel filtration HPLC procedure, indicating that the mature toxin is oligomeric. This was indeed confirmed by quick-freeze and deep-etch electron microscopy (EM) (Lupetti *et al.*, 1996; Cover *et al.*, 1997). Extremely rapid freezing prevents the distortion of the molecules which may occur with other techniques, thus allowing the mould to accurately represent the surface of the molecule. VacA appears as a flower with either six or seven petals (Fig. 2A; Lupetti *et al.*, 1996). These electronic images were used to derive a three-dimensional structure (Fig. 2B) of the toxin by a method akin to tomography (Lanzavecchia *et al.*, 1998). Although this technique exhibits a relatively low resolution (around 30 Å) compared with crystallography, it provides an accurate picture of the outer part of the molecule, and thus gives some insights into its molecular architecture. More recently, the P58 subunit was purified and subjected to similar structural studies (Reyrat *et al.*, 1999). It was shown that the P58 moiety, when expressed in an homologous genetic context without the P37 subunit, is able to fold into a soluble structure with remarkable similarity to the peripheral petals of the holotoxin oligomer. This has led to a better understanding of the contribution of each building block to the overall structure of the toxin, and has suggested a model in which the monomers are intercalated with each other to form the ring structure. Hence, the oligomeric structure may be maintained by interaction between the P37 subunit of one monomer and the P58 subunit of the adjacent monomer (Fig. 2C). It has, however, been shown that activation at acidic pH results in the dissociation of the toxin into monomers (Cover *et al.*, 1997; Molinari *et al.*, 1998a; Reyrat *et al.*, 1998), suggesting that oligomeric VacA has a very low activity. Cover *et al.* (1997) also detected groups of up to 12 monomers in EM studies of acid-treated VacA and have suggested that the oligomeric structure is bilayered. Although the measurements of the molecule obtained from the three-dimensional reconstructions (Lanzavecchia *et al.*, 1998; Reyrat *et al.*, 1999) are not compatible with dodecameric structures, it cannot be excluded that under some conditions the hexameric structures shown in Fig. 2C may associate to form bilayered dodecameric structures.

The role of the connective loop, i.e. the region where processing occurs, has been investigated by constructing derivatives in which the loop was either shortened by 16–46 amino acids or substituted by the loop of the toxin of *Corynebacterium diphtheriae* (Burroni *et al.*, 1998). Interestingly, although the toxic activity was unaffected, the shortening of the loop appeared to favour an organization into hexamers. It is likely that the shortening of the connective loop between the P37 and the P58 pulls these subunits closer together, which reduces the flexibility of the interaction and consequently the number of monomers that can enter into the oligomeric structure.

VacA toxicity

There is a strong consensus that VacA is actually a cytotoxin. Indeed, when administered orally to mice (Telford *et al.*, 1994), VacA induces gastric epithelial erosion resembling that observed in humans with peptic ulcer. *In vitro* studies have demonstrated that VacA binds to target cells in a dose-dependent and saturable manner which is not increased by exposure to acid pH (Massari *et al.*, 1998). It is then internalized into endosomes with kinetics compatible with receptor-mediated endocytosis (Garner and Cover, 1996). A 140 kDa protein (Yahiro *et al.*, 1997) and the epidermal growth factor (EGF) receptor (Seto *et al.*, 1998) have been successively proposed as VacA receptors, but definitive evidence is still lacking. Recently, the toxin binding function has been ascribed to the P58 moiety because this subunit binds to target cells. In the absence of P37, with the same affinity as the wild-type toxin without inducing any vacuolization (Garner and Cover, 1996; Reyrat *et al.*, 1999). However, binding of P58 was not followed by its internalization in

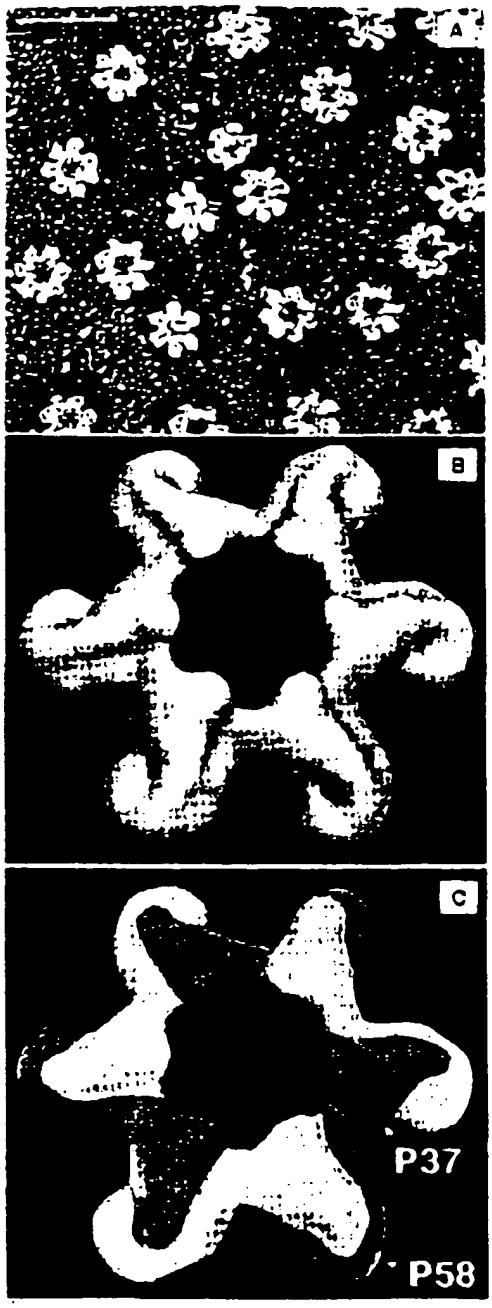

Fig. 2. A. Electron micrograph of purified VacA (bar represents 50 nm).
B. Three-dimensional top view of wild-type VacA replicas.
C. Model of the possible interaction between 87 kDa monomers in the oligomeric structure. In the hypothetical model, alternate monomers in the oligomeric structure are shaded differently in order to represent the interaction of the P37 subunit of one monomer with the P58 subunit of the adjacent monomer. P58, 58 kDa subunit; P37, 37 kDa subunit; dotted line, connecting loop between P58 and P37.

cells, suggesting that the P37 is also needed for efficient endocytosis (Reyrat *et al.*, 1999). However, the exact definition of VacA toxicity *in vitro*, which varies with the model system used, and th *in vivo* relevance of these various biological properties are rather elusive. Available information allow the ranking of known toxic effects into the three classes listed below.

Dysfunctions of the endocytic pathway

VacA was first described to induce vacuolation of mammalian cells *in vitro* (Leunk *et al.*, 1988). This consists in the cytoplasmic accumulation of large prelysosomal compartments, marked by the late endosomal, small GTP-binding protein Rab7 (Papini *et al.*, 1994, 1997) and by the lysosomal protein Lgp 110 (Molinari *et al.*, 1997). Vacuolation inhibition by V-ATPase blockers (Cover *et al.*, 1993; Papini *et al.*, 1993) and by overexpression of non-functional *rab7* alleles (Papini *et al.*, 1997) suggest that modifications of both osmotic and membrane traffic balances at the prelysosomal level underlie vacuole biogenesis. The complexity of vacuole generation is demonstrated by the fact that it can be deeply influenced by cell type, density, differentiation and serum concentration in the culture medium (de Bernard *et al.*, 1998a). Cytosolic expression of the *vacA* gene was shown to induce a *rab7*/V-ATPase/weak base-dependent vacuolation of HeLa cells (de Bernard *et al.*, 1997). The entire amino-terminal domain of VacA (P37) plus a short sequence belonging to the P58 domain were sufficient to induce vacuolation (de Bernard *et al.*, 1998b), proving that this activity, whether enzymatic or not, is contained within this portion and can be exerted from the cytosol. These results were recently confirmed and extended (Ye *et al.*, 1999). Full vacuolation of HeLa cells can be obtained by expressing the P37 moiety together with a 166-amino-acid fragment of the P58 subunit as separate molecules. However, it is still unclear whether both fragments contain essential parts of a putative catalytic core of the toxin. It is worth noting that the connective loop is at the interface between these two fragments, suggesting that the essential determinant of toxin activity is not contiguous along the protein sequence (Ye *et al.*, 1999). However, it is currently unknown whether the 166-amino-acid fragment of the P58 domain is part of a functional toxic domain or whether it is required to assist folding and oligomerization of the P37 subunit. Another important result of these studies was that the toxin expressed in the cell cytosol was fully active even in the absence of acid pH pretreatment. Studies with model membranes suggest that exposure to acid pH, leading to disassembly of the inactive toxin oligomer into monomers, may increase the insertion of the toxin into lipid bilayers (Molinari *et al.*, 1998a; Czajkowsky *et al.*, 1999) and hence trigger the hydrophobic interaction of VacA with the cell plasma membrane, the first step of any following cellular effect.

It should be noted that macroscopic vacuolation is preceded by extracellular mistargeting of lysosomal hydrolases.

decreasing cytosome–lysosomal degradative power in HeLa cells (Satin *et al.*, 1997). These *in vitro* observations may have clinical relevance *in vivo*. In fact, Molinari *et al.* (1998b) have shown that interference with membrane trafficking results in an inhibition of antigen presentation to the immune system. Such effects are likely to reflect early stages of VacA action on the endocytic pathway, which include a partial neutralization of the lumen (Satin *et al.*, 1997).

Permeability changes in polarized epithelial cell monolayers

In vitro treatment of polarized epithelial monolayers with VacA, or with VacA-expressing *H. pylori* strains, increases the transepithelial diffusion of ions (recorded as a decrease in TER – transepithelial electrical resistance) and small neutral tracers with molecular weight lower than 450 Da (Papini *et al.*, 1998; Pelicic *et al.*, 1999). When toxin-producing bacteria were used, VacA present on the surface of the bacteria did not require acidic treatment to exert its activity on epithelia (Pelicic *et al.*, 1999). Quite surprisingly, this effect, most likely due to a modulation of cell–cell junctions, occurs in the virtual absence of endocytic alterations and, in contrast to vacuolation, is not inhibited by BafA1 and not enhanced by ouabain or weak bases (Papini *et al.*, 1998). It has been proposed that a selective diffusion of essential ions such as Ni^{2-} and Fe^{3+} and low-molecular-weight nutrients (glucose, amino acids) from the underlying mucosa toward infecting *H. pylori* may represent an advantage for efficient gastric colonization (Papini *et al.*, 1998).

Channel formation in lipid bilayers

Experiments conducted with artificial lipid bilayers show that VacA activated by acidic pH forms low-conductivity, voltage-dependent, anion-selective channels (Iwamoto *et al.*, 1999; Tombola *et al.*, 1999). Selectivity analysis showed that the channels conduct Cl^- and HCO_3^- and, although less efficiently, the organic carboxylic acids pyruvate and gluconate. The VacA channel is blocked by the typical chloride channel blocker DIDS (4,4'-dilsothiocyanatostilbene-2,2'-disulphonic acid) (Iwamoto *et al.*, 1999). Moreover, VacA is able to interact with the lipid bilayer and form structures compatible with hexameric pores that can be visualized by atomic force microscopy and that probably account for the observed chloride channel activity (Czajkowsky *et al.*, 1999; Iwamoto *et al.*, 1999). However, in spite of this similarity, there is no identifiable sequence homology between VacA and any known chloride channels (Iwamoto *et al.*, 1999). Interestingly, the dimeric P58 was unable to form channels in lipid bilayers, demonstrating that the presence of the P37 is not only required for efficient endocytosis (Reyrat *et al.*, 1999) but also for pore formation (Tombola *et al.*, 1999). It is tempting to speculate that the P58 dimeric molecule may lack the channel activity due to the dimeric structure which does not allow the formation of the large central ring. Once formed in the apical membrane of polarized epithelia, channels may account for the observed toxin-induced increase of Isc (short circuit current) in $CaCo_2$ epithelial monolayers (Guarino *et al.*, 1998) and possibly for *H. pylori*-associated diarrhoea in children (Sullivan *et al.*, 1990). Endocytosis and delivery of a sufficient amount of VacA channels to endosomes is expected to increase the turnover of the electrogenic V-ATPase by augmenting anion permeability of the membrane of these compartments (Tombola *et al.*, 1999). Excess protons in the endosomal lumen would combine with available weak bases to generate osmotically active species. Water influx would hence cause endosome swelling. Further studies are necessary to substantiate this model in a cellular system and to prove a relationship among channel formation by VacA, vacuole generation and also changes of TER (Montecucco *et al.*, 1999).

Mosaicism and geographical variation of VacA

H. pylori isolates not cytotoxic for HeLa cells are often identified (Cover *et al.*, 1994), but they almost invariably contain the *vacA* gene and, surprisingly, the corresponding protein is expressed in most cases. Little attention was initially paid to this class of isolates which were infrequently reported in the early literature, and they were thus called tox$^-$. However, it is becoming clear that m2 strains are more frequent in Western countries than originally thought. It has been shown that this lack of vacuolating activity is associated with an allelic variation occurring in the mid-region of the gene, more precisely in the P58 subunit (Cover *et al.*, 1994; Atherton *et al.*, 1995, 1997; Wang *et al.*, 1998b). The two alleles are known as m1 and m2 and antisera raised against the m1 form of the protein poorly recognize the m2 form (Pagliaccia *et al.*, 1998). Furthermore, the m2 form has been systematically associated with a lack of cytotoxicity in HeLa cells (Atherton *et al.*, 1995). However, this did not fit with the observation that both m1 and m2 isolates are involved in the development of severe forms of gastric disease, thus pointing to the toxic character of the m2 strain (Go *et al.*, 1998). Recently, by expressing the *vacA* gene intracellularly in HeLa cells, it was shown that m2 cytotoxins are indeed active, strongly suggesting that the lack of *in vitro* activity is due to defects in some early step(s) of HeLa cell intoxication (Pagliaccia *et al.*, 1998). Using a combination of biochemical and genetic techniques, it was shown that m2 cytotoxins vacuolate RK-13 cell lines and human epithelial cells, indicating that their lack of activity in HeLa cells

resulted from a binding defect (Pagliaccia *et al.*, 1998). Hence, the m2 toxins are indeed active, as active as the m1, and confirmed that the m region within the P58 subunit is involved in cell binding. The intrinsic activity of m2 toxins was again confirmed when entire bacteria were tested in the TER model described above (Pelicic *et al.*, 1999), suggesting that the cell monolayers are likely to have both kind of receptors needed to interact with m1- and m2-type toxins.

The ratio of m1/m2 strains differs in different regions of the world: m1 isolates are found more frequently (>80%) in South America, Portugal and Spain (Atherton *et al.*, 1995, 1997; van Doorn *et al.*, 1999), whereas m2 strains are prevalent in China (Pan *et al.*, 1998). Surprisingly, in Japan, a close neighbour of China, m1 strains are predominant (Ito *et al.*, 1997; Shimoyama *et al.*, 1998). In northern Europe and North America, about 50% of strains are m1. It is likely that in those countries where both alleles are present, there is frequent recombination between m1 and m2 alleles in the population (Suerbaum *et al.*, 1998). In support of this, another variant of the mid-region has been described, although with a very low prevalence, in which the m region is a half m1 region and half m2 region hybrid (Atherton *et al.*, 1998; Pan *et al.*, 1998) and most probably arose from intragastric recombination between m1 and m2 bacterial populations during mixed infections that are indeed frequently found. Untypable mid-region populations have also been described and have been shown to cluster, indicating a further subfamily of *vacA* alleles (Strobel *et al.*, 1998). This allelic variation may represent a coevolution between the toxin and human ecotypes (Covacci *et al.*, 1999).

In addition, it has been recently reported that, in a few cases, true toxin strains do exist and possess a silent *vacA* gene or carry mutations in the gene such as internal duplications, large deletions, or small insertions that cause truncation of the VacA toxin (Ito *et al.*, 1998). These inactivated toxin genes are considered to be cryptic genes which can be reactivated by mutation. This microdiversity in *vacA* genetic sequences reflects the global diversity and plasticity of the *H. pylori* genome (Marshall *et al.*, 1998).

Another type of mosaicism concerning the VacA signal peptide has been described previously (Atherton *et al.*, 1995, 1997). This classification offers only a limited amount of information concerning toxic activity because the signal peptide is removed during secretion. Four alleles, namely s1a, s1b, s1c and s2, have been identified. Thus, toxins may exist with differing combinations of the signal peptide and the mid-region, although only one strain with a s2m1 combination has been reported so far (Letley *et al.*, 1999). The s2 signal peptide seems to be defective and consequently leads to a small release of secreted toxin (Atherton *et al.*, 1995, 1997; Cover, 1996). Consequently, people infected by an s2 toxin-producing strain will develop only mild forms of gastric disease, such as gastritis (Atherton *et al.*, 1997; van Doorn *et al.*, 1998). s2 variants are found less frequently and do not seem to have a particular geographical repartition.

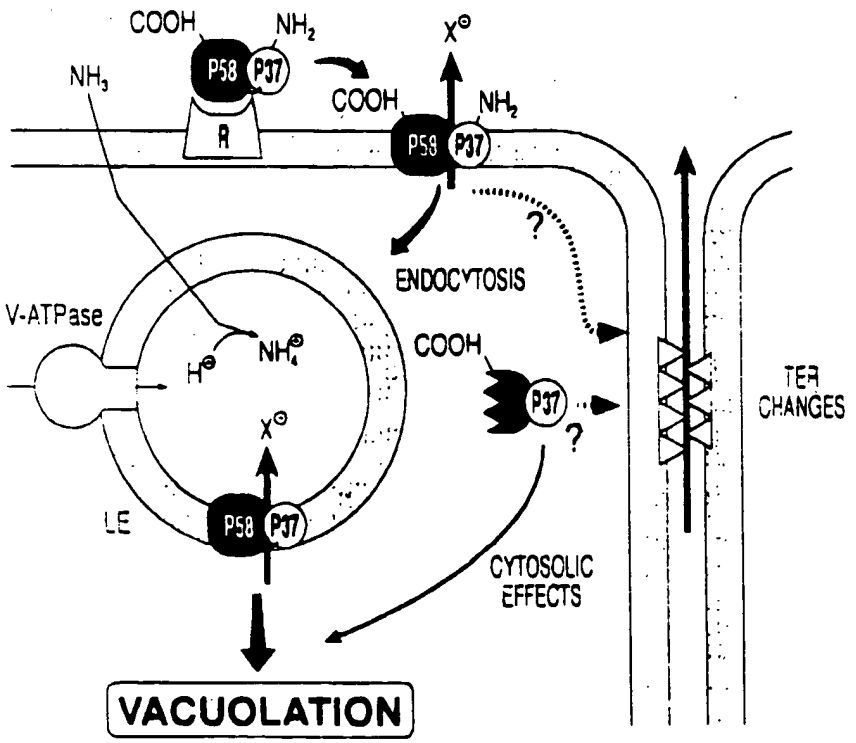

Fig. 3. Hypothetical model of cell intoxication by VacA. The toxin binds, via its carboxyl-terminal domain (P58), to an unknown receptor (R) on the apical portion of epithelial cells. Active monomeric toxin inserts into the plasma membrane via hydrophobic protein–lipid interactions. The insertion step, which also requires the amino-terminal domain (P37), results in the formation of an anion selective channel. Endocytosis of the toxin and transport to endosomes, another P37-dependent step, is proposed to increase the anionic permeability of these compartments and in turn enhance the vacuolar ATPase proton pumping activity. In the presence of weak bases, in particular the ammonia generated by the *H. pylori* urease, the endosomal accumulation of osmotically active acidotropic ions (NH_4^+) is predicted to increase, leading to water influx and vesicle swelling, two essential steps in vacuole formation. Plasma membrane-associated channels may also trigger, by a still unknown secondary mechanism, changes in the paracellular permeability of polarized epithelial cell monolayers. Alternatively, active VacA delivered in the cytosol, formed by P37 and a region of P58, could act on cell–cell junctions modifying the TER.

Concluding remarks

A large amount of research has now been carried out on all aspects of VacA biology. Genetic studies have shown that the toxic activity is localized mostly in the P37 domain, whereas the binding activity is localized in the P58 subunit. Although the cleavage into the P37 and P58 fragments and the binding activity of the P58 domain relates VacA to the AB-type family, the functional data do not fully support this because a small portion of the P58 is needed to achieve vacuolation. Therefore, in the absence of a formal demonstration of enzymatic activity and identification of the cytosolic target, this classification remains somehow elusive. Caution is also suggested by the fact that endocytosis after surface binding requires the putative active domain (P37), which might interact with some other co-receptor responsible for toxin internalization. Moreover, P58 alone lacks pore-forming activity, a property which, again, requires the presence of P37. Based on these data, it is tempting to speculate that VacA possesses an interesting and perhaps unique molecular structure as it exhibits structural and intoxication features of both AB and pore-forming toxins. Several toxic activities are now known, however the definition of the intracellular target remains a main challenge for future research. It is still puzzling whether vacuolation, monolayer TER decrease and gastric atrophy represent different manifestations of the same toxic activity, or whether they correspond to different activities on different intracellular targets. The recent discovery that VacA forms channels at very low doses in artificial lipid bilayers, extended to cellular systems, may simplify to some extent the interpretation, linking vacuole formation to the ability of VacA to increase anion permeability at the level of plasma membrane and endosome (Fig. 3). However, the picture is still incomplete because a connection with the TER changes is so far missing. In conclusion, VacA is clearly a key player in the development of peptic ulcer, and further understanding of its toxicity will require pharmacological, electrophysiological and cellular microbiology approaches.

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EXHIBIT C

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Establishment of Gastric *Campylobacter pylori* Infection in the Neonatal Gnotobiotic Piglet

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Campylobacter pylori, a gram-negative microaerophilic bacterium, has been implicated in the genesis of human gastritis, dyspepsia, and gastroduodenal ulceration. Previous attempts to reproduce the diseases in conventional laboratory animal species have been unsuccessful. To determine if neonatal gnotobiotic piglets were susceptible to *C. pylori*, we orally challenged two litters ($n = 17$) with 10^9 CFU after pretreating them with cimetidine. Controls housed in separate units received nothing or peptone water alone. Piglets were examined 1, 2, 3, and 4 weeks after challenge. Colonization by the bacterium and inflammation of the gastric mucosa persisted throughout the study period. Organisms were revealed by Warthin-Starry silver stain to reside between the mucus layer and the gastric epithelium. Culturing of samples from sites along the gastrointestinal tract revealed that the bacterium colonized essentially only the gastric and proximal duodenal mucosae. Gross pathological changes were restricted to the stomachs of infected piglets and consisted of submucosal edema, increased gastric mucus production, and progressive development of mucosal lymphoid follicles. Microscopic lesions consisted of transient neutrophilic infiltrates followed by diffuse and follicular infiltrations of mononuclear leukocytes into the mucosa and submucosa. Alcian blue–periodic acid-Schiff stains suggested that the infection resulted in the depletion of mucopolysaccharide production by deep gastric glands. These data indicate that gnotobiotic piglets reproduce many of the features of diseases associated with *C. pylori* in humans.

Chronic gastritis and attendant clinicopathologic entities, such as nonulcerous dyspepsia and gastroduodenal ulceration, are commonly encountered in human gastroenterology (3, 6). The etiology and pathogenesis of these conditions are unclear but have been linked to living conditions of high stress characteristic of western society, diet, and possibly duodenogastric bile and acid reflux. Symptoms include indigestion, sternal or epigastric discomfort, generalized abdominal pain, burping, gastric distention, and halitosis (6). Numerous nonprescription drugs, chiefly antacids and antifoaming agents, are available, and affected individuals are encouraged by media advertising to indulge in self-treatment regimens with these products.

An emerging candidate agent for the genesis of gastritis or ulceration is a gram-negative spiral bacillus, *Campylobacter pylori*, first cultured from gastric mucosa in 1984 (17). Previous workers noted the occurrence of "spirochetes" in gastric tissues, but disease associations were not made (for a review, see reference 6). Since that initial report from Australia, a number of investigators have reported similar findings (2, 11, 21–24), although not all are in agreement as to their pathologic significance (8, 14). In attempting to link lesions to disease processes, most authors emphasize the importance of concurrent histopathologic examination of gastric biopsies (6, 15). When these studies are performed carefully with the understanding that gastritis associated with *C. pylori* is a focal or multifocal lesion, the correlation of lesions with successful bacterial isolations is high (1, 6, 15).

Prospective clinical, pathologic, and immunologic investigations are needed to confirm or deny the role of *C. pylori* in human gastritis. Clinical investigations into naturally occurring diseases in humans have the disadvantages of patient selection, development of inclusive (and exclusive) diagnostic criteria, design of a therapeutic regimen(s), and compliance with prescribed treatment. Oral infection of human volunteers obviates many of these problems but brings with it expense and limited flexibility in the design and execution of microbiologic and pathologic studies. What is clearly needed is an animal model system in which disease factors can be easily manipulated and the limitations inherent in the use of experimental human subjects can be avoided.

To this end, oral challenge of numerous adult and neonatal laboratory animal species, including mice, rats, rabbits, guinea pigs, and germfree rats, has been performed (6; D. R. Morgan, unpublished data); all have been unsuccessful. All of these species have the disadvantage of a gastrointestinal system notably different from that of humans. The pig is a functional monogastric mammal with dietary habits and anatomical and physiological characteristics similar to those of humans (9, 12). Thus, the primary objective of this study was to determine if gnotobiotic piglets are susceptible to oral challenge with *C. pylori*. Specifically, we wished to determine whether gastric infection is established and what the microbiologic and pathologic consequences of gastritis induced by *C. pylori* are.

(This work was presented in part at the annual meeting of the Infectious Disease Society of America in New Orleans, La., on 3 October 1986.)

MATERIALS AND METHODS

Animals. A total of 17 gnotobiotic domestic Yorkshire piglets from two litters (8 and 9 per litter) were used in this study. Both litters were derived from date-mated pregnant sows by Caesarian section essentially by procedures described previously (25). Neonatal piglets were transferred into sterile pentub isolation units containing six partitions, an exterior heat source was applied, and the piglets were fed

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FIG. 1. Submucosal lymphoid follicles in the mucosa and submucosa of a piglet challenged orally with *C. pylori* 32 days previously.

a diet of Similac plus iron per os three times daily (100 to 150 ml per feeding). Uninfected controls were maintained in separate isolation units.

Preparation of bacterial inoculum. Forty-eight-hour broth cultures of *C. pylori* were used to prepare the challenge inoculum. Bacteria were grown in brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% fetal bovine serum and 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) in 50-ml Erlenmeyer flasks. Flasks were placed in GasPak (BBL Microbiology Systems) jars with a CampyPak (BBL Microbiology Systems) to provide an appropriate atmosphere, and the jars were shaken on a gyratory platform (120 rpm) at 37°C. Cultures were harvested by centrifugation, suspended in peptone water, and enumerated by standard plate counting before challenge.

Microbiology. Daily fecal swabs for bacterial cultures were taken from all piglets in litter 1. Swab samples were taken from the surfaces of the cardium, fundus, pylorus, and duodenal bulb in experiment 1. In experiment 2, swab samples were taken from the stomach regions, oropharynx, esophagus, duodenum, jejunum, ileum, spiral colon, terminal colon, and rectum. Samples were inoculated onto GCHI agar plates supplemented with trimethoprim, vancomycin, polymyxin B sulfate, and amphotericin B (Remel, Lenexa, Kans.) for bacterial isolation. The plates were incubated at 37°C in a GasPak jar for 3 to 7 days. Isolates were identified as *C. pylori* on the basis of Gram stain reaction and xidase, catalase, and urease production.

Experimental design. At 4 and 5 days of age, piglets were rendered temporarily achl rhydric by oral administration of cimetidine (60 mg/kg). Infected piglets received a suspension of *C. pylori* containing 10^9 CFU in 2.0 ml of peptone water orally on day 5 of age after a 12-h fast. Controls received no treatment (litter 1) or peptone water alone (litter 2).

The infection studies were conducted by litter in two experiments. Piglets were examined three times daily for clinical signs of disease. In experiment 1 (eight piglets), six piglets were challenged with 3.8×10^9 CFU of *C. pylori* and two piglets were used as controls. Piglets were euthanized and examined 1 (two infected and one control), 2 (two infected), and 3 (two infected and one control) weeks after challenge. At necropsy, the stomachs were opened along the longitudinal axis, and swab and tissue samples were collected from the above-mentioned four anatomical regions. Gross lesions, when present, were noted.

In experiment 2 (nine piglets), six piglets were challenged with 4.4×10^9 CFU of *C. pylori* as described above and three piglets were used as negative controls. Piglets were euthanized and examined 1, 2, and 4 weeks after challenge (two infected and one control at each interval).

Histopathology. Tissue samples for histopathology were taken from sites adjacent to those sampled for microbiology. In experiment 1, samples were fixed in 10% neutral buffered Formalin. In experiment 2, the stomachs of one infected piglet and the uninfected control piglet from each interval were removed by ligation and resection and inflated with a fixative composed of 4% (v l/vol) Formalin and 1% (vol/vol) glutaraldehyde in a phosphate buffer (4F1G) formulation (18). After fixati n, tissue samples were embedded in paraffin and sectioned as 6-μm pieces, and replicates were

FIG. 2. Rare small aggregate of lymphoid cell elements in the lamina propria of the pylorus in an uninfected control piglet. Hematoxylin-eosin stain.

ained with hematoxylin-eosin, Warthin-Starry (WS) silver, d Alcian blue–periodic acid-Schiff (PAS) stains.

Serology. At necropsy, a terminal serum sample was ollected from each anesthetized piglet and frozen for the ub quent determination of *C. pylori* antibody. Sera were ested for the development of antibody by a standard en- yme-linked immunosorbent assay. Formalinized broth- ultured *C. pylori* (10^7 CFU per well) was used as the antigen microtiter plates. Alkaline phosphatase-conjugated affin- y-purified goat anti-swine immunoglobulin G (Bio-Rad aboratories, Rockville Centre, N.Y.) was used as the condary reagent for pig antibody.

RESULTS

Clinical signs and macroscopic lesions. Infection did not ult in grossly visible gastric epithelial erosions or ulcer- ons. An increase in luminal gastric mucus in infected us control piglets was especially prominent from 2 eks after challenge on. Other tissues, including the intes- were unremarkable. In experiment 1, four of six in- ted piglets exhibited mild transient diarrhea 2 days after llenge; one piglet was listless and mildly anorectic 2 to 4 after challenge. In experiment 2, similar results were ained; several infected piglets exhibited transient ano- ia. Prominent submucosal and mucosal lymphoid follicles dules) were seen in both infected piglets sacrificed 4 eks after challenge (Fig. 1).

Microscopic lesions. Hist pathologic lesi ns indicative of ronic active gastritis were n ted in all piglets infected with *pylori*. The intensity and severity of these changes creased with time. For c nvenience, lesions detected in th experiments are described together. Microscopic changes in uninfected controls were quantitatively similar throughout and consisted of occasional mononuclear cells found in the submucosa of the gastric fundus and pylorus. Rare small submucosal lymphoid aggregates were seen (Fig. 2). Neutrophils were never observed.

One week after challenge with *C. pylori*, gastric cardiac regions contained neutrophilic infiltrates largely restricted to the nonglandular (i.e., epithelial) regions of the cardia. Neutrophils formed intraepithelial aggregates (microabscesses) and were also present in the lamina propria. Mononuclear leukocytes were also detected in this region but were more prominent in the nonglandular regions. Aggregates of cells were present in the submucosal regions and occasionally obliterated crypt regions. Small lymphoid follicles were detected in the cardia.

Two weeks after challenge, the neutrophilic response had resolved. There was a notable increase in the number of mononuclear cells in both the submucosa and the lamina propria. In the latter regions, discrete lymphofollicular aggregates were apparent. Three weeks after challenge, the microscopic lesions had intensified, chiefly because of the infiltration and proliferation of mononuclear cells. Lymphoid follicles were prominent and occasionally coalesced to form large sheets of cells encompassing both the submucosa and the lamina propria (Fig. 3).

The Alcian blue-PAS histochemical stain is useful in determining the ability of gastric epithelia to secrete various general classes of mucopolysaccharides. A deep-blue-staining product (Alcian blue) is considered indicative f acid mucopolysaccharide (AMP) producti n, whereas a PAS-positive reaction product (red) stains predominantly neutral mucopolysaccharides (NMPs). Each of th four anatomical regions of the stomach was evaluated for types

FIG. 3. Lymphoid follicle development in the submucosa and lamina propria of a gnotobiotic piglet 20 days after challenge with *C. pylori*. Hematoxylin-eosin stain.

and ratios of stained cells at the luminal (i.e., superficial gastric mucus-secreting epithelium) and deep glandular (mucus-secreting cell) pits. Tables 1 and 2 summarize these findings.

Controls showed essentially the same pattern of reaction over time. At the luminal epithelial surface, AMP production predominated in the cranial (cardia and fundus) regions, whereas the frequency and intensity of NMP production increased in the pylorus and duodenal bulb. In gastric pits, the opposite reaction pattern was observed. That is, in the cardia and fundus, NMP production predominated, whereas in the distal regions, AMP production predominated.

In infected piglets, there was a reduction in AMP-positive luminal cells in the anterior regions of the stomach in the later stages (2 and 4 weeks after challenge) f infection, as compared with controls; distal staining patterns remained unchanged and were indistinguishable from c ntrol staining patterns. Staining patterns in gastric glands in infected piglets were indistinguishable from those in controls 1 week after challenge. However, 2 and 4 weeks after challenge, there was a marked reduction in and depletion of both AMP and NMP production by deep gastric glands in sections of the fundus and pylorus. These regions correspond to those in which histopathologic lesions associated with *C. pylori* infection were most prominent.

Microbiology. Table 3 summarizes the results of both WS silver staining and bacterial isolation from the stomachs of infected and control piglets in both experiments. Organisms were recovered from at least one anatomical region from all infected piglets at each examination interval after challenge. In experiment 2, organisms were observed in or recovered from the esophagus of tw piglets (1 and 4 weeks after challenge) and in the duodenum of tw piglets (2 and 4 weeks after challenge). In these instances, h wever, the tissues did n t exhibit the hist pathologic lesions observ d in the stomach, and it is most likely that these isolation data reflect

TABLE 1. Alcian blue-PAS staining patterns in sections of gastric mucosa from the three uninfected control gnotobiotic piglets of litter 2

Section and piglet no.	Cell ratio[a] in indicated region:			
	Anterior		Distal	
	Cardia	Fundus	Pylorus	Duodenal bulb
Luminal surfaces				
86-3229	10:1	4:1	1:10	1:10
86-3227	4:1	1:4	1:10	1:10
86-3228	10:1	1:4	1:10	1:10
Gastric glands				
86-3229	1:10	1:10	1:1	4:1
86-3227	1:10	1:10	1:1	10:1
86-3228	1:10	1:10	1:1	10:1

[a] Data are expressed as the ratio of Alcian blue (AMP)-positive to PAS (NMP)-positive mucus-secreting cells on luminal surfaces and in gastric glands.

spillover from the stomach which occurred during the manipulations performed for gastric ligation and resection. Organisms were not recovered from the feces.

Organisms were not revealed in controls by WS silver staining. In contrast, organisms were revealed by WS silver staining in all infected animals in at least one of the four anatomical regions throughout the 4-week study period. Bacteria were largely restricted to the superficial mucus-secreting layer of the gastric epithelium (Fig. 4). Organisms were extraepithelial in location and appeared to be attached to the glycocalyx of the cells beneath the acellular mucus layer. Occasionally, organisms were noted in the deeper portions of the mucosa. As before, organisms appeared to be extracellular and were restricted to the lumina of occasional dilated gastric pits. Structurally intact organisms were not seen in the submucosa or lamina propria.

Serology. Specific antibody to *C. pylori* was present in sera from challenged animal from 2 weeks after challenge on (Table 4). Animals sacrificed 1 week after challenge and all control animals, irrespective of the time of sacrifice, had no serum antibody specific for *C. pylori*.

DISCUSSION

The data reported in these experiments demonstrated that neonatal gnotobiotic piglets were susceptible to oral challenge and subsequent gastric colonization by *C. pylori*. Infection appeared to be largely restricted to the stomach and persisted for longer than 4 weeks after oral challenge. The organism was not shed in a viable form in the feces and did not appear to colonize other, nongastric segments of the intestinal tract. Infected piglets were largely asymptomatic. Microscopic lesions, if present, were subtle, correlating well with human gastritis associated with *C. pylori*. Gastric infection resulted in the development of characteristic microscopic lesions which were first detected as a transient and then persistent neutrophilic infiltration into the nonglandular cardia. Subsequent lymphocytic gastritis of the glandular portion of the stomach progressed from focal accumulations of cells in the submucosa and lamina propria to the formation of discrete lymphoid follicles located in both the submucosa and lamina propria. The WS silver stain was useful in identifying the site(s) of colonization within the stomach. Most of the organisms were located extracellularly on the superficial (luminal) epithelial surface between the epithelial cells and the superficial protective mucus layer. This tropism corresponds to similar sites of colonization in humans (6, 7, 15). When compared with controls, piglets infected with *C. pylori* exhibited histochemical changes in mucopolysaccharide production by gastric mucus-secreting epithelium. Grossly, this was manifested as an apparent increase in free mucus in the stomach. Histologically, this was manifested chiefly as a reduction in the amounts of mucopolysaccharides in the deep gastric glands, which correlates with the depletion of the mucus layer associated with human gastritis (5). The Alcian blue-PAS stain is not quantitative and is useful only in identifying trends. Clearly, more precise biochemical measures of these changes are needed before definitive statements regarding the effects of infection upon mucus production can be made.

The pattern of histologic lesions observed was remarkably similar to that observed in humans with gastritis associated with *C. pylori* (6, 15, 23). Others have emphasized neutrophilic infiltrates in affected human gastric mucosa (6, 15). In piglets, neutrophilic responses were transient, inconsistent, and largely restricted to the nonglandular cardia. A consistent finding in humans is a reduced mucus content in mucosal cells (6). Similar changes were apparent in tissue sections examined 4 weeks after challenge. It is likely that further reductions in mucus production would have occurred with time, thereby ultimately mimicking human lesions. This phenomenon in piglets remains to be determined, however.

The development of specific antibody to *C. pylori* after challenge is consistent with a true infection by this bacterium. Humans with gastric *C. pylori* infections produce specific antibody and, in this way, the porcine model parallels human disease. Serum antibody may be a useful diagnostic indicator of human *C. pylori* infections (1, 8, 10, 16).

Except for transient intraepithelial abscesses and attendant microscopic epithelial erosions in the nonglandular (cardiac) portion of the stomach, ulceration of the gastric

TABLE 2. Alcian blue-PAS staining patterns in sections of gastric mucosa from the gnotobiotic piglets of litter 2 infected with *C. pylori*

Section, day postinfection, and piglet no.	Cell ratio[a] in indicated region:			
	Anterior		Distal	
	Cardia	Fundus	Pylorus	Duodenal bulb
Luminal surfaces				
7				
86-3221	10:1[a]	4:1	1:10	1:10
86-3222	10:1	4:1	1:10	1:10
14				
86-3223	4:1	1:1	1:10	1:10
86-3224	4:1	4:1	1:10	1:10
32				
86-3225	10:1	1:1	1:10	1:10
86-3226	1:10	0[b]	1:10	1:10
Gastric glands				
7				
86-3221	1:5	1:5	1:1	4:1
86-3222	1:10	1:10	1:1	10:1
14				
86-3223	1:10	—[c]	—	3:1
86-3224	1:10	—	—	3:1
32				
86-3225	1:10	—	1:10	4:1
86-3226	1:10	1:10	1:1	10:1

[a] See Table 1, footnote *a*.
[b] 0, A relevant area(s) of the mucosa on the microslide was missing.
[c] —, Depletion (i.e., a complete lack of an AMP or NMP reaction product).

TABLE 3. Microbiological findings in the gastric anatomical regions of gnotobiotic piglets infected with *C. pylori*

Piglet group and time after challenge	No. of piglets from which a viable culture was obtained/total no. examined for indicated region				No. of piglets in which organisms were revealed by silver staining/total no. examined for indicated region			
	Cardia	Fundus	Pylorus	Duodenal bulb	Cardia	Fundus	Pylorus	Duodenal bulb
Uninfected controls (n = 5)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Infected piglets								
1 wk (n = 4)	2/3	2/3	2/3	2/3	3/4	3/4	3/4	3/4
2 wk (n = 4)	3/3	2/3	3/3	2/3	4/4	2/4	2/4	3/4
3 wk (n = 2)	2/2	2/2	2/2	2/2	2/2	1/2	2/2	1/2
4 wk (n = 2)	1/1	1/1	1/1	1/1	0/2	2/2	2/2	2/2

glandular mucosa in infected piglets was not observed. Among domestic animal species, pigs are noted for the spontaneous development of gastric ulceration and perforation (4, 9, 19, 20). In a sense, this syndrome is incorrectly named, since the ulcers are usually restricted to the nonglandular (esophageal or cardiac) region of the organ. Although the pathogenesis is unknown, naturally occurring ulceration is seen under conditions of performance stress (feeder and finishing operations) and in association with low-roughage diets high in digestible protein, unsaturated fat, and carbohydrates. Diets of this nature are thought to predispose pigs to ulceration by inducing the overseer

FIG. 4. *C. pylori* in the superficial mucus-secreting layer of the gastric epithelium in a piglet 6 days after challenge with *C. pylori*. WS silver stain.

TABLE 4. Antibody titers to *C. pylori* in sera of gnotobiotic control piglets and piglets challenged with *C. pylori*

Piglet no.	Sacrifice interval (wk)	Reciprocal titer[a] of *C. pylori*-specific immunoglobulin G
Litter 1		
86-1918 (Control)	1	5
86-1917 (Control)	3	5
86-1914	1	5
86-1915	1	5
86-1913	2	10
86-1916	2	80
86-1911	3	80
86-1912	3	80
Litter 2		
86-3229 (Control)	1	5
86-3227 (Control)	2	5
86-3228 (Control)	4	5
86-3221	1	5
86-3222	1	5
86-3223	2	5
86-3224	2	5
86-3225	4	80
86-3226	4	160

[a] Dilution factor of pig serum in the last well which produced a positive reaction (defined as twice the background).

of acid (via the release of histamine or serotonin) and delaying the emptying of the stomach (9). These factors are thought to disturb the natural pH gradient within the stomach, thereby permitting the reflux of acid cranially to effect a mucus-free and thus unprotected cardiac or esophageal epithelium. Support for this hypothesis is engendered by studies which have demonstrated that the oral administration of prostaglandin inhibitors (e.g., indomethacin) indirectly enhances the relative acidity within the organ by reducing the prostaglandin-mediated formation and release of bicarbonate by gastric mucus cells (9). Experimentally, ulceration can be produced in pigs by parenteral administration of histamine and reserpine (19, 20).

In humans, the pathogenesis of gastroduodenal ulcers is poorly understood (1). An inappropriate production of gastric acid is thought to be the main precipitating cause of this malady (6). Support for this concept comes largely from clinical observations which indicate that drugs which antagonize acid secretion, namely, cimetidine and ranitidine, promote the healing of ulcers. There can be no doubt that continued acid secretion promotes ulceration after initiation in the gastric mucosa. In an otherwise normal individual, it is difficult to envision a situation in which the overproduction of acid per se is a primary event. It is more likely that acid production is an important cofactor that perhaps functions to perpetuate and accentuate ulceration induced by other mechanisms. Gastritis induced by *C. pylori* may be the important and unrecognized initiator of this common human condition.

In summary, we have shown that neonatal gnotobiotic piglets are susceptible to infection by a human pathogen, *C. pylori*. The organism colonizes gastric mucosa and reproduces many features of the corresponding disease syndrome in humans. Further development and exploitation of this nonprimate animal model should permit the discovery of the pathogenic mechanisms of gastric disease associated with this agent and should ultimately provide insights into a more rational approach to the diagnosis and treatment of human gastric disease.

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ADDENDUM

Since the original submission of our manuscript, Lambert and co-workers (13) published preliminary data for one litter of neonatal gnotobiotic piglets demonstrating gastric colonization and inflammation following pretreatment with ranitidine and oral challenge with 10^6 CFU of *C. pylori*. A similar challenge of conventional, colostrum-deprived piglets failed to result in gastric colonization or inflammation. Recent experiments in our laboratories demonstrated that challenge of conventional, colostrum-deprived piglets with 10^9 CFU of *C. pylori* also failed to result in gastric colonization or inflammation (manuscript in preparation).

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EXHIBIT D

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Helicobacter pylori Gastric Infection in Gnotobiotic Beagle Dogs

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Establishment of infection with *Helicobacter pylori* and gastritis in nonhuman species is currently only successful in gnotobiotic piglets. This study was designed to determine whether *H. pylori* will colonize the gastrointestinal tract of gnotobiotic dogs. Gnotobiotic beagle pups were derived by standard methods. Group A (five dogs) was orally challenged with 3×10^8 *H. pylori* at 7 days of age. Group B (two dogs) received only peptone water but was contact-exposed beginning on day 23 postinfection (p.i.). Necropsy was performed on dogs on day 30 p.i. *H. pylori* colonized the stomach of all dogs (groups A and B). Urease map analysis correlated with the microbiologic findings and indicated that the density of colonization was less than that observed in human tissue. Organisms were also recovered from the pharynx, esophagus, duodenum, and rectum of 1, 2, 2, and 1 dog, respectively. All group A and one group B dog developed serum immunoglobulin G specific for *H. pylori* by day 30 p.i. Gross lesions were restricted to the stomach and consisted of small (<1 mm) lymphoid follicles. Microscopically, there were focal to diffuse lymphoplasmacytic infiltrates with follicle formation and mild to moderate infiltration of neutrophils and eosinophils in the gastric lamina propria. With the Warthin-Starry silver stain, organisms were seen on the surface of the gastric epithelial cells, beneath the mucus layer. We conclude that *H. pylori* colonizes the stomachs of gnotobiotic dogs for at least 1 month and the lesions resemble those seen in humans. *H. pylori* is transmissible by contact from infected to noninfected dogs.

Helicobacter pylori (formerly *Campylobacter pylori*) is a gram-negative microaerophilic bacterium that causes gastritis and is associated with nonulcer dyspepsia and gastroduodenal ulcer in humans (6, 18). Oral challenge of volunteers results in histologic lesions of chronic active gastritis as well as symptoms of dyspepsia (19, 20). Treatment of *H. pylori*-associated gastritis with combinations of antimicrobial agents, bismuth, and H-2 antagonists in humans has met with limited success, and recurrence is frequent (10). Because of this, the use of human volunteers to study this disease entity is not practical, and adequate models for study of the pathogenesis of this disease are needed.

H. pylori will not colonize many of the usual laboratory animal species, including conventionally reared rats, mice, rabbits, guinea pigs, specific-pathogen-free pigs, colostrum-deprived piglets, and gnotobiotic rats and mice (9; Krak wka et al., Second Int. Symp. on *C. pylori*, in press). We and others have shown that the gnotobiotic neonatal piglet is susceptible to oral infection with *H. pylori* (15, 16). In this model, infection is limited to the stomach, and the lesions that develop are those of lymphoplasmacytic gastritis, resembling human infection (5). The gnotobiotic pig model, however, is limited because of the inability to maintain pigs in the gnotobiotic state for greater than 45 to 60 days because of size and nutritional constraints. In addition, study of ulcerogenesis may be confounded by the susceptibility of weanling pigs to the development of ulcers induced by diet and stress (3). The advantages of a dog model include the ability to maintain the animals in the gnotobiotic condition for years, the availability of well-established methods for studying immunologic and gastric physiologic responses, and the lack of propensity to develop spontaneous ulcers. The objective of this study was to determine whether gnotobiotic dogs are susceptible to gastric infection by *H. pylori*.

MATERIALS AND METHODS

Animals. A litter of seven gnotobiotic beagle pups was derived from specific-pathogen-free bitches by standard methods (14). They were maintained in sterile Pentub isolation units and fed a diet of Esbilac (PatAg, Inc., Hampshire, Ill.). Initially, unchallenged control dogs were housed separately from inoculated dogs. Beginning on day 23 postinfection (p.i.), controls were housed together with infected dogs to determine whether infection would spread via contact.

Bacterial inoculum. A virulent strain of *H. pylori*, 26695, was used. This is a human isolate and is capable of colonizing and producing gastritis in gnotobiotic piglets (7). Bacteria were grown in 250-ml Erlenmeyer flasks containing brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% fetal calf serum (BBL Microbiology Systems, Cockeysville, Md.). Flasks were incubated at 37°C in a 10% CO_2 atmosphere on a rotary shaker at 150 rpm. Cultures were harvested by centrifugation 24 h after inoculation (logarithmic growth phase), washed, and suspended in peptone water. Organisms were enumerated with a hemacytometer and by standard plate count. An inoculum of 3×10^8 CFU in 2.0 ml of peptone water was prepared.

Experimental design. At 7 days of age, five pups (group A) were orally challenged with 3×10^8 CFU of *H. pylori* in 2.0 ml of peptone water. Group B (two pups) served as controls and were given peptone water alone. Group B was initially housed separately from their infected littermates. On day 7 p.i., two pups from group A and one from group B were gavaged, and the stomach contents were cultured for reisolation of *H. pylori*. In order to ascertain whether contact infection was possible, the dogs in group B were subsequently housed with their infected littermates beginning on day 23 p.i. Blood samples were collected prior to challenge

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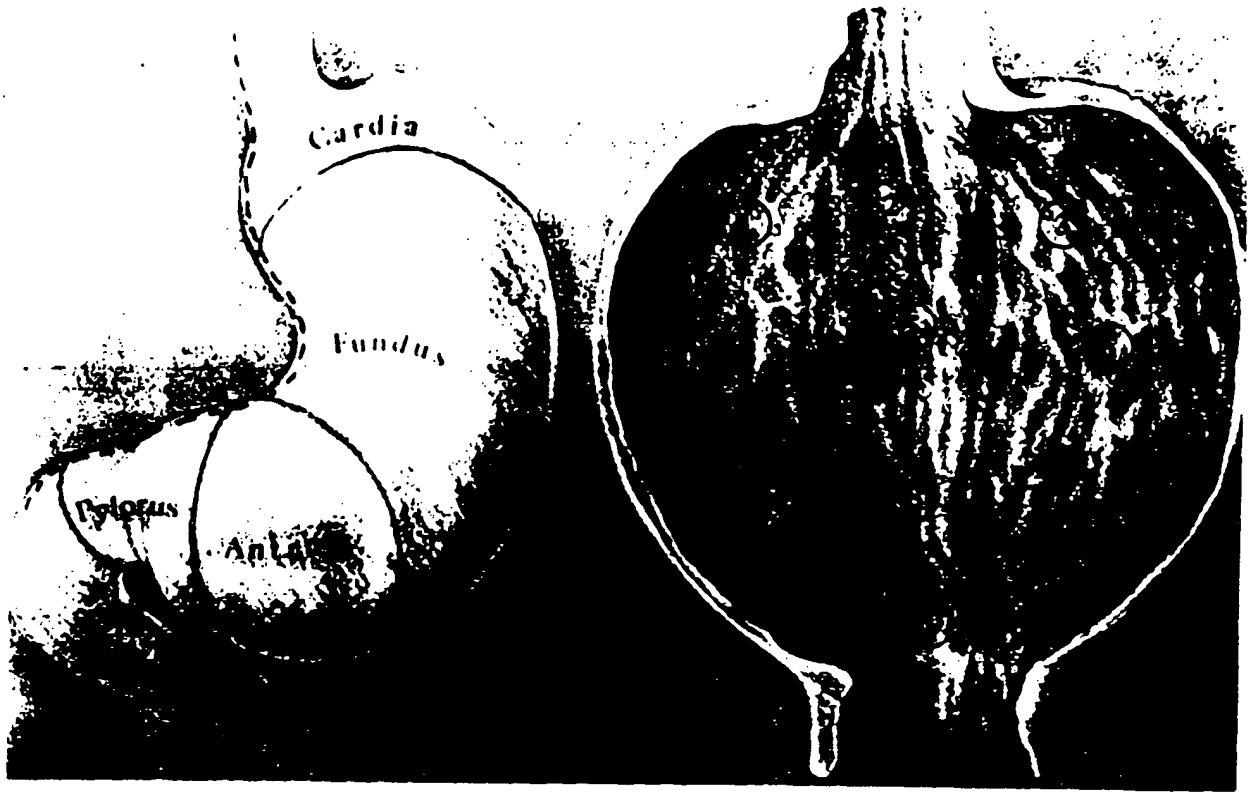

FIG. 1. Sites of biopsy for urease mapping (see Table 4).

and then once weekly until the conclusion of the study. The serum was frozen at −20°C and saved for serologic study. On day 30 p.i., the pups were anesthetized with 3.5 mg of xylazine and 17.5 mg of ketamine hydrochloride, and a pH electrode was passed into the stomach to measure the gastric pH in vivo. After this, the pups were killed with an overdose of pentobarbital.

Mucosal samples were obtained from the entire gastrointestinal tract, including the pharynx, esophagus, stomach (cardia, antrum, fundus, and pylorus), duodenum, jejunum, ileum, colon, and rectum. The gastrointestinal tract was examined for gross lesions, and samples for histopathologic examination were taken from the same regions as for culture. Multiple punch biopsies were taken from the stomach for urease mapping. Scrapings of the gastric mucosa were examined for organisms by phase-contrast microscopy.

Urease mapping. Urease mapping was performed to determine the distribution and to estimate the density of colonization by *H. pylori* in the stomach (12). Three punch biopsies each were taken from the cardia, fundus, and antrum of the stomach; one sample was taken from the pylorus (Fig. 1). The biopsy samples were incubated for 24 h in sealed microtiter plate wells containing urea, phenol red, and sodium azide in sodium phosphate buffer, pH 6.5. A positive test was detected by color change (from orange to dark pink) of the medium, and time until the change occurred was recorded. The time to positivity of this test has been shown to be proportional to the number of bacteria present at the biopsy site (12).

Microbiology. Samples taken of stomach contents by lavage on day 7 p.i. and musocal samples taken from the gastrointestinal tract at the completion of the study were streaked on blood agar (BBL Microbiology Systems, Cockeysville, Md.) and incubated for 5 to 7 days in a 10% CO_2 atmosphere. Swab samples of the isolator surfaces and samples of the food were similarly cultured. Plates were examined for growth after 7 days, and density of infection was estimated by counting the number of colonies per plate (estimated colony counts).

Histopathology. Samples taken for histologic evaluation were fixed in 10% neutral buffered Formalin and embedded in paraffin. Sections (6 μm) were stained with hematoxylin and eosin for histopathologic evaluation and with Warthin-Starry silver stain for identification and localization of the bacteria.

Serology. *H. pylori* isolated at the time of necropsy was grown for 4 days on 5% sheep blood agar in a 10% CO_2 atmosphere and used to prepare antigen for an enzyme-linked immunosorbent assay (ELISA). Bacteria were harvested in phosphate-buffered saline (PBS) containing 0.02% sodium azide (pH 7.4), washed three times, and disrupted by sonication. The preparation of antigen contained 9.5 mg of protein per ml and was stored at 4°C until use.

Antigen was diluted in 0.1 M carbonate buffer (190 μg of protein per ml, pH 9.6), and 100 μl was added to each well of flat-bottomed polystyrene ELISA plates (Immulon II; Dynatech, Chantilly, Va.). Plates were incubated at 4°C for 16 h to allow coating and then washed three times with wash buffer (PBS containing 0.05% Tween 20 and 0.1% nonfat dried milk). A blocking step was performed by incubating the wells with 200 μl of wash buffer at 37°C for 1 h, followed by aspiration of the wash buffer. Sera from the dogs were diluted in PBS containing 0.05% Tween 20 and added to each well (100 μl). Plates were incubated at 37°C for 1 h and then

TABLE 1. Microbiological findings in the four regions of the stomach and other areas of the gastrointestinal tract of gnotobiotic dogs infected with *H. pylori*[a]

Dog group and no.	Growth of *H. pylori* in culture										
	Stomach				Gastrointestinal tract						
	Cardia	Fundus	Antrum	Pylorus	Phar	Esoph	Duod	Jejun	Ileum	Colon	Rectum
Group A											
89-1021	+	+	+	+	+	+	−	+	−	−	−
89-1022	+	+	+	+	−	+	−	−	−	−	
89-1023	+	+	+	+	+	−	−	−	−	−	
89-1024	+	+	+	+	−	+	−	−	−	−	
89-1025	+	+	+	+	−	+	+	−	−	−	−
Group B											
89-1026	+	+	+	+	−	−	−	−	−	−	−
89-1027	+	+	+	+	+	+	+	−	−	+	−

[a] Growth (+) or no growth (−) from mucosal tissue samples after 7 days on blood agar in a 10% CO_2 atmosphere. Phar, Pharynx; Esoph, esophagus; Duod, duodenum; Jejun, jejunum.

washed three times. Affinity-isolated alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin G (IgG; Sigma Chemical Co., St. Louis, Mo.) was diluted 1:500 in wash buffer, and 100 μl was added to each well for 1 h (37°C). Wells were washed three times, and 100 μl of substrate (5-mg tablet of *p*-nitrophenyl phosphate dissolved in 10 ml of 10% diethanolamine [pH 9.6]; Sigma Chemical Co., St. Louis, Mo.) was added. After 30 min, optical density was read at 420 nm (OD_{420}) (reference, 570 nm), and samples showing an OD_{410} of ≥0.14 units were considered positive.

RESULTS

Microbiology. *H. pylori* colonized the stomachs of all group A dogs (Table 1). Culture of stomach contents obtained by gavage on day 7 p.i. resulted in recovery of *H. pylori* from the two group A dogs but not from the unexposed group B dogs. However, after 7 days of exposure to infected dogs, both group B dogs became colonized (Table 1), demonstrating that infection may be transmitted by contact. All four regions of the stomach were colonized in both groups. In addition, *H. pylori* was recovered from the pharynx, esophagus, duodenum, jejunum, and colon of some pups (Table 1). Organisms were not recovered from the isolator environment, fecal material, or food.

The degree of colonization as judged by estimated colony count was greatest in the stomach, with the fundic region tending to be the most heavily colonized (Table 2). Of the contact-exposed dogs, one was heavily colonized and one was lightly colonized in the stomach. The remainder of the gastrointestinal tract was lightly or not colonized.

Clinical signs and necropsy findings. At no time during the study did the dogs appear ill. Gastric pH was variable in the group A dogs and ranged from 1.1 to 6.4. The gastric pH of group B dogs was low (pH 1.4 and 1.9) (Table 3). In contrast to infected humans and gnotobiotic pigs, the dogs did not exhibit excess gastric mucus production. Gastric ulceration did not occur, and gross lesions, when present, were mild and consisted of multiple small (<1.0 mm) lymphoid follicles in the stomach. It was possible to demonstrate the bacterium under phase microscopy in all but one dog.

Urease mapping. Urease production was most pronounced in the fundic area (Table 4), which was also the most heavily colonized site. Unlike in humans, in whom the time until detectable urease-induced color change can be as short as 10 min, biopsy samples from the dogs required several hours for color development to occur, suggesting that bacterial colonization in the dog is not as heavy as in humans. Urease activity was not detectable in lightly colonized areas (<10 colonies per plate).

Histopathology. All dogs had chronic active gastritis characterized by focal to diffuse lymphoplasmacytic cellular infiltrates in the lamina propria. Lymphoid follicle formation occurred (Fig. 2) and was most prominent in the antrum. Neutrophils and eosinophils were often associated with the lymphoid follicles (Fig. 3) and were also lightly scattered along blood vessels and the basal border of the lamina propria of all regions of the stomach. Rare pockets of neutrophils and eosinophils were seen. No gastric epithelial erosions or ulcerations were observed. In contrast, lesions of chronic active gastritis and infiltration of the lamina propria with neutrophils or eosinophils have not been observed in uninfected age-matched gnotobiotic beagle dogs maintained under similar conditions and on a similar diet in our laboratory (M. J. Radin and S. Krakowka, unpublished data).

Inflammation was mild in the small intestine. Neutrophils and eosinophils were scattered in the lamina propria of the duodenum (four and one dog from groups A and B, respectively) and jejunum (all dogs). Follicular hyperplasia of the tonsils and ileal Peyer's patches was seen and probably represents a general response to antigenic stimulation. Both group B dogs had very mild neutrophilic-lymphocytic esophagitis. The group B dog with a positive colonic culture also had mild lymphoid follicle development in the colon.

TABLE 2. Estimated colony counts from the four regions of the stomach and gastrointestinal tract of gnotobiotic dogs infected with *H. pylori*

Dog group and no.	No. of colonies[a]				
	Cardia	Fundus	Antrum	Pylorus	GI tract
Group A					
89-1021	100–200	>500	50–100	50–100	<10
89-1022	100–200	50–100	50–100	<10	<10
89-1023	50–100	>500	>500	100–200	<10
89-1024	100–200	50–100	<10	<10	<10
89-1025	>500	>500	>500	<10	<10
Group B					
89-1026	<10	<10	<10	<10	0
89-1027	200–500	>500	>500	10–50	<10

[a] Number of colonies that grew on a plate after streaking with similar amounts of mucosal samples. GI, Gastrointestinal.

TABLE 3. Gross observations for gnotobiotic dogs infected with *H. pylori*

Dog group and no.	Lymphoid follicles[a]	Excess luminal mucus[a]	Gastric pH	Organisms[b]
Group A				
89-1021	−	−	3.3	−
89-1022	−	−	6.4	+
89-1023	1	−	1.6	+
89-1024	3	−	2.2	+
89-1025	1	−	1.1	+
Group B				
89-1026	−	−	1.9	+
89-1027	3	−	1.4	+

[a] Increasing severity of lesion from − to 3.
[b] Organisms present (+) or absent (−) in gastric mucosal scrapings observed by phase-contrast microscopy.

TABLE 4. Results of gastric urease mapping of the stomach of gnotobiotic dogs infected with *H. pylori*

Dog group and no.	Presence of urease at stomach site[a]									
	1	2	3	4	5	6	7	8	9	10
Group A										
89-1021	+	−	−	+	+	+	+	−	+	+
89-1022	+	−	+	+	+	+	−	−	−	−
89-1023	+	−	+	+	+	+	+	−	+	−
89-1024	+	−	+	+	+	−	−	−	−	−
89-1025	+	−	+	+	+	+	−	−	−	−
Group B										
89-1026	−	−	−	−	−	−	−	−	−	−
89-1027	+	−	+	+	+	+	+	+	+	+

[a] Presence (+) or absence (−) of urease based on color change after 24 h of incubation. Sites: 1 to 3, cardia; 4 to 6, fundus; 7 to 9, antrum; 10, pylorus (see Fig. 1).

H. pylori was demonstrated in all regions of the stomach by Warthin-Starry silver staining. Bacterial colonization occurred on the surface of the stomach and appeared to be heaviest in the gastric pits. The bacteria were closely associated with the apical surface of the gastric epithelial cells, beneath the mucus layer (Fig. 4). Bacteria were not detected in Warthin-Starry-stained sections in any other area of the gastrointestinal tract.

Serology. Four group A dogs developed serum IgG specific for *H. pylori* by day 14 p.i., and all seroconverted by day 30 p.i. (Table 5). The heavily colonized group B pup had a titer of 1:200 on day 30 p.i., indicating that seroconversion may occur as early as 1 week after exposure. The second contact-exposed pup did not have detectable IgG at the end of the experiment, which may be related to the light colonization observed in this animal.

DISCUSSION

H. pylori colonizes the stomach of gnotobiotic dogs following oral challenge. The infection persists for at least 1 month after inoculation and, as in the gnotobiotic pig model

FIG. 2. Lymphoid follicle formation in the lamina propria of a gnotobiotic dog 30 days after oral challenge with *H. pylori*. Hematoxylin-eosin stain.

FIG. 3. Infiltration of neutrophils and eosinophils adjacent to a lymphoid follicle in the lamina propria of a gnotobiotic dog 30 days after oral challenge with *H. pylori*. Hematoxylin-eosin stain.

(15), appears to be asymptomatic. It should be noted that colonization of the gnotobiotic dog stomach as determined by urease mapping and histopathologic examination is not as marked as in human patients (12) or gnotobiotic piglets (15). In addition, the distribution of the infection is different, with the fundic mucosa being predominantly colonized in the dog versus the antrum in humans. This is consistent with the hypothesis that the human is the primary host of *H. pylori*.

In contrast to pigs and humans infected with *H. pylori*, in which colonization is restricted to the stomach, *H. pylori* was recovered by culture from other areas of the gastrointestinal tract of the gnotobiotic dog. We cannot entirely exclude the possibility that *H. pylori* reisolated from sites other than the stomach represents organisms transiently passing through the gastrointestinal tract. However, because of the presence of associated mild microscopic lesions in the esophagus, duodenum, jejunum, and colon in the dogs, the possibility of colonization of other regions of the gastrointestinal tract must be considered.

The lesions of chronic active gastritis in the dogs resembled those seen in humans (5, 18, 22). Like humans, the dogs develop a lymphoplasmacytic gastritis with continued infiltration f neutrophils. This differs from the gnotobiotic piglet, in which the neutrophilic infiltration is transient (15), and may suggest that infectious gastritis in the dog more cl sely resembles the human conditi n. Unlike in gnotobiotic pigs, gastritis in the dogs was associated with a mild eosinophilic infiltrate in the areas of inflammati n. Eosinophilic infiltration has been reported in acute *H. pylori* gastritis in humans (8, 20). Eosinophilic inflammation is more often associated with parasitic infestation or allergic responses than with bacterial infection in both dogs and humans. These pups were derived from specific-pathogen-free bitches on a strict deworming program, so it is unlikely that the presence of eosinophils was the result of larval migration. An allergic response to other environmental antigens is also unlikely. Age-matched gnotobiotic pups reared under similar conditions do not have eosinophilic infiltrates or gastritis. It is probable that the presence f these inflammatory cells represents an aspect of host immune response to *H. pylori*.

Macroscopic lesions in the dogs, when present, were mild, which is similar to the case of gnotobiotic piglets and the majority of humans with *H. pylori*-associated gastritis. Ulceration was not observed. The pathogenesis of gastroduodenal ulceration in humans infected with *H. pylori* is probably multifactorial (10). It is likely that interaction of other environmental promoting agents, such as nonsteroidal anti-inflammatory drugs, smoking, and alcohol, with *H. pylori* gastritis may be required for ulcerogenesis. The gnotobiotic dog model should provide a system by which these factors may be tested.

Most of the pups had a fasting gastric pH of less than 3.0; one pup had a gastric pH of 6.4. By 5 weeks of age, normal beagle dogs tend t maintain a resting gastric pH of 3 or less and are capable of responding to histamine and pentagastrin

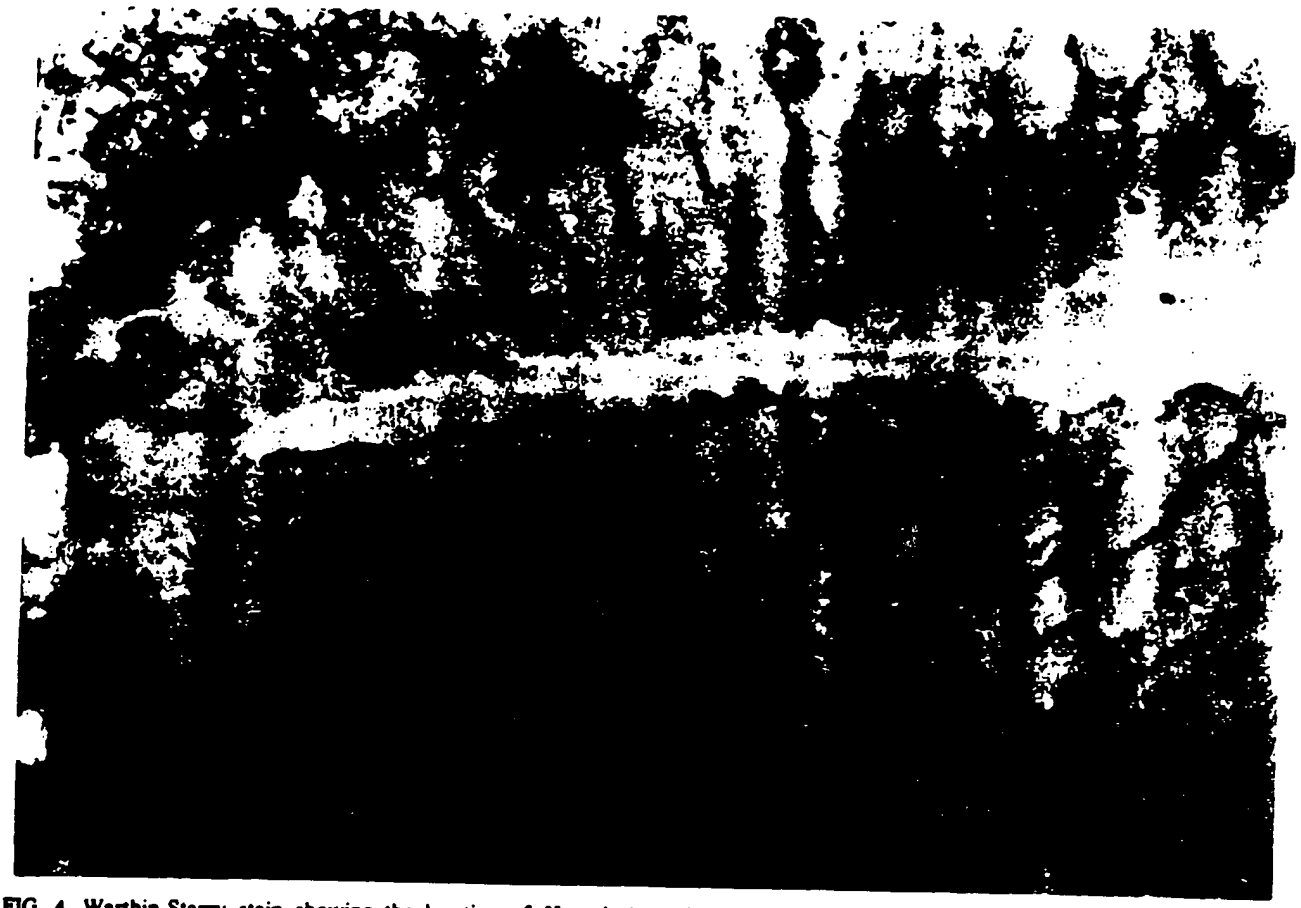

FIG. 4. Warthin-Starry stain showing the location of *H. pylori* on the surface of the gastric epithelial cells in a gastric pit of a contact-exposed gnotobiotic dog.

17). *H. pylori* infection has been associated with transient ypochlorhydria in humans (11, 20, 21). Neutralization of astric acid or inhibition of acid production by the bacteria ay be an integral part of the disease syndrome. Recent udies suggest that *H. pylori* is capable of inhibiting acid cretion of parietal cells in vitro and that this inhibition may quire attachment of *H. pylori* to gastric epithelial cells (2, . Studies with humans have shown that *H. pylori* may exist th- stomach under a variety of gastric acid secretory ate-, and the time sequence and importance of hypochlor- ydria is unknown (1). In the gnotobiotic dogs, the gastric pH measured 1 or 3 weeks after challenge was not correlated to either severity of histologic lesions or number of bacteria recovered.

It is unknown how *H. pylori* is transmitted or what the source is for reinfection of treated patients. Our data show that *H. pylori* is transmissible by contact from infected to uninfected dogs. The mechanism by which this transmission occurs was not determined and may be either oral-oral or fecal-oral. The presence of mild esophagitis in the two contact-exposed dogs suggests that colonization rostral to the stomach plays a role in early infection and transmission of *H. pylori*. In a recent survey, *H. pylori* was isolated from dental plaque of one human patient with concurrent gastritis. (13). Further work is needed in this area.

Most of the group A pups had detectable specific IgG titers for *H. pylori* by 2 weeks postchallenge. Seroconversion may take place as early as 1 week after exposure, as seen in the one contact-exposed dog, and may be related to the severity or duration of colonization. This rapid rise in serum IgG is in contrast to that seen in humans, in whom 3 or more weeks may be required to produce a detectable IgG response (8, 20). As in humans, rising IgG titers in dogs were n t accompanied by clearing of the infection.

In conclusi n, *H. pylori* will persistently colonize the gastric muc sa of gnot biotic d gs for at least 1 month. The resultant disease syndrom resembles the human condition, with the producti n of chronic active gastritis and serocon-

TABLE 5. Serum IgG antibody response to *H. pylori* over time as measured by ELISA

group and no.	IgG (ELISA titer) on day p.i.:			
	0	14	21	30
up A				
-1021	—[a]	—	1:50	1:50
-1022	—	1:200	1:100	≥1:400
-1023	—	1:25	—	1:25
-1024	—	1:50	1:50	1:200
-1025	—	1:50	1:20	≥1:400
up B				
-1026	—	—	—	—
-1027	—	—	—	1:200

[a] Not detectable.

version. In addition, *H. pylori* may be transmitted by c ntact from infected to uninfected dogs. Despite the apparent differences in degree and distribution of colonization compared with humans, our data indicate that the gnotobiotic d g may provide a good model for the study of therapeutic regimens as well as strategies for th prevention of transmission and early colonization by this human pathogen.

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EXHIBIT E

Brief Definitive Report

Gene Structure of the *Helicobacter pylori* Cytotoxin and Evidence of Its Key Role in Gastric Disease

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Summary

The gram negative, microaerophilic bacterium *Helicobacter pylori* colonizes the human gastric mucosa and establishes a chronic infection that is tightly associated with atrophic gastritis, peptic ulcer, and gastric carcinoma. Cloning of the *H. pylori* cytotoxin gene shows that the protein is synthesized as a 140-kD precursor that is processed to a 94-kD fully active toxin. Oral administration to mice of the purified 94-kD protein caused ulceration and gastric lesions that bear some similarities to the pathology observed in humans. The cloning of the cytotoxin gene and the development of a mouse model of human gastric disease will provide the basis for the understanding of *H. pylori* pathogenesis and the development of therapeutics and vaccines.

The recently discovered, gram negative, microaerophilic bacterium *Helicobacter pylori* colonizes the human gastric mucosa and establishes a chronic infection that is tightly associated with atrophic gastritis, peptic ulcer, and gastric carcinoma (1–5). *H. pylori* infection is a worldwide problem, since in developing countries it affects over 80% of the population older than 20. Also in developed countries the infection is present in 20% of the population by the age of 30 rising to over 50% by the age of 60. Clinical isolates of *H. pylori* can be classified into two groups based on the presence or absence of the vacuolating cytotoxin (6, 7) whose expression is linked to a surface exposed immunodominant antigen (CagA) (8, 9). Since high titers of serum antibodies to the CagA protein are detected in all patients with duodenal ulcer (8) and most of those with gastric carcinoma (10, 11), it has been proposed that disease development requires infection with cytotoxin-producing strains.

The cytotoxin causes massive vacuolation in several mammalian cell lines (6), and similar vacuoles have also been observed in the gastric epithelia of patients with active chronic gastritis associated with *H. pylori* infection (12), indicating that the cytotoxin can contribute significantly to the pathogenesis of gastritis. Cell vacuolation in vitro can be blocked and reversed by inhibitors of V-type ATPases and potentiated by inhibitors of the Na^+-K^+ ATPase (13, 14), suggesting that the mechanism of action of the toxin is due to aberrant cation transport within the target cells. The purified toxin has been described as a protein of ~87 kD that is found in the bacterial culture supernatants, and the sequence of the NH_2-terminal 23 amino acids has been determined (7).

Despite the epidemiological correlation between infection with cytotoxic strains and disease (8) and the in vitro evidence for the presence of a cytotoxin, the in vivo roles of infection and cytotoxin have not been established due to the lack of a suitable animal model. *H. pylori* does not colonize the gastric mucosa of mice or other small laboratory animals. To overcome this limitation, we administered *H. pylori* extracts and purified cytotoxin orally to mice. Using this model, extracts from cytotoxic *H. pylori* strains and purified cytotoxin induced a gastric pathology with some similarities to that observed in *H. pylori*–associated human disease.

In addition, we have cloned the gene coding for the cytotoxin responsible for the gastric lesions and determined the nucleotide sequence. Antisera against recombinant fragments of the toxin were used to study the synthesis and processing of the protein.

Materials and Methods

Purification of the Cytotoxin. Toxic activity was concentrated from *H. pylori* culture supernatant by precipitation with 50% ammonium sulphate, recovered in 20 mM sodium phosphate buffer, pH 7.0, and applied to a CM-Sepharose CL-6B column. The toxin was eluted with a gradient of 0–0.5 M NaCl. SDS-PAGE of frac-

tions eluted at ~150 mM NaCl indicated purification to h mogeneity of a 94-kD protein that was recognized by antisera raised against recombinant fusion proteins. The purified material contained no detectable urease either by immunoblot using urease-specific antibodies or by enzymatic activity (15).

Treatment of Mice. 6-wk-old BALB/c mice were deprived of food, but allowed free access to water. After 24 h the mice received an administration of saline alone (0.5 ml) or containing 5 μg of purified cytotoxin or 100 μg of *H. pylori* sonic extract. The samples were introduced via catheter inserted into the eosophagus to ensure delivery. The administration was repeated after 48 h. After a further 48 h, the mice were killed and portions of the gastric mucosa were fixed in 4% formalin and embedded in paraffin. 7 μm sections were cut, rehydrated, and stained in hematoxilin and eosin.

Gene Cloning and Sequencing. *H. pylori* DNA was prepared and plasmid libraries were prepared as described previously (8). Two oligonucleotide mixtures were prepared: one containing all possible combinations of the codons coding for the amino acid sequence AFFTTV and one complementary to all combinations of the sequence GTAVGT. These mixes were used at a concentration of 4 μM with 100 ng of total *H. pylori* DNA in a polymerase chain reaction with Taq polymerase. The reaction was cycled through 1 min at 94°C, 2 min at 48°C, and 2 min at 72°C 30 times. The product of the degenerate PCR was labeled by random priming and used to screen a library of HindIII restriction fragments cloned in the bluescript SK(+) plasmid. An ~3 kbp fragment was isolated that contained the first 273 bp of the coding sequence. A 120-bp fragment was derived from the 3-kb fragment by digestion with EcoRI and was used to screen a library of EcoRI fragments. Clones containing an ~7-kb overlapping fragment were isolated. The nucleotide sequence of the coding region and flanking regions of the gene were determined using the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH). Sequence analysis programs used were Wordsearch, FastA, and Peptidestructure as implemented in Version 7 of the Genetics Computer Group sequence analysis package.

Expression of Cytotoxin Fragments. DNA fragments containing the sequences coding for amino acids 34–132 (region A), amino acids 262–428 (region B), amino acids 731–1,000 (region C), amino acids 1,001–1,121 (region D), and amino acids 1096–1220 (region E) were prepared either by PCR or restriction enzyme digestion of the cloned gene and cloned, in frame, in the appropriate version of the expression vector pex34 (16). Fusion proteins were extracted from *Escherichia coli* harboring the plasmids as described (17) and purified by SDS-PAGE before the immunization of rabbits.

Preparation of H. pylori Proteins. Total proteins were extracted from *H. pylori* cells using 6 M guanidinium hydrochloride as described (8). Culture supernatants were concentrated by precipitation with 50% ammonium sulphate. The precipitate was resuspended in phosphate buffered saline and dialyzed against the same buffer.

Protein Sequencing. Protein fragments were electroeluted after PAGE, applied to ProSpin tubes and sequenced in a pulsed liquid protein sequencer (model 477A; both from Applied Biosystems, Inc., Foster City, CA).

PCR Analysis of Cytotoxin Negative Strains. Based on the sequence shown in Fig. 2, oligonucleotides of length 20 were prepared to amplify the following regions of the sequence: Nucleotides 1–780, 332–934, 768–1,464, 1,468–2,692, 2,646–3,693, 3,259–3,925, and 3,861–4,087. The products of PCR using the cloned gene, total DNA from three cytotoxic strains (CCUG 17874, 60190, G39), and three cytotoxin negative strains (TX30, G21, G50) were analyzed by agarose gel electrophoresis.

Results

A Model of H. pylori–induced Ulceration. Oral administration in mice of a sonicate of a cyt toxin producing strain of *H. pylori* caused epithelial vacuolation and infiltration of mononuclear inflammatory cells in the lamina propria (Fig. 1 *b*). The vacuolation of cells seen in the proximity of the more severe epithelial lesions has also been observed in patients suffering from *H. pylori* associated active chronic gastritis (12) and can be induced by the cytotoxin in cells in vitro (6). Occasional ulceration with substitution of the mucosal tissue with early stage granulation tissue and loss of gastric gland structure was observed (Fig. 1 *c*). Administration of saline did not cause any significant mucosal damage (Fig. 1 *a*). Administration of sonic extract from a noncytotoxic strain of *H. pylori* (G21) did not cause epithelial lesions and resulted in gastric histology essentially identical to that shown in Fig. 1 *a*. Thus, in this model, we have produced erosive lesions in the murine gastric mucosa and confirmed the association of these lesions with cytotoxin producing strains.

Administration of vacuolating cytotoxin, purified to homogeneity, caused similar epithelial lesions in the absence of extensive inflammation. Of six mice treated with purified toxin, all revealed localized regions of cell necrosis accompanied by loss of cytoplasm and general loss of gastric gland architecture (Fig. 1 *d*). In two of the six mice, gastric ulceration was also observed (Fig. 1, *e* and *f*). The ulceration was characterized by focal mucosal injury and the presence of inflammatory exudate within the healing tissue. Of 10 control mice and 6 mice administered with 5 μg of purified *H. pylori* urease, all showed normal gastric histology (data not shown). We conclude that the cytotoxin alone is responsible for most *H. pylori*–induced epithelial erosion.

The Cytotoxin Gene. To isolate the gene coding for the cytotoxin, two oligonucleotide mixtures were prepared corresponding to the sequences capable of coding for the first 6 amino acids and complementary to the last 6 amino acids of the 23 known NH_2-terminal amino acids (7). The mixtures were completely degenerate at the third base of every codon. A polymerase chain reaction was carried out with total DNA from strain CCUG 17874 of *H. pylori* and a fragment of the expected size was obtained. The PCR product was used to probe a library of *H. pylori* DNA digested with HindIII and cloned in the bluescript plasmid vector. Clones containing an ~3-kbp insert were isolated. Clones containing an ~7-kbp insert that overlapped with 120 bp of the HindIII clone were subsequently isolated from a library of EcoRI restriction fragments. These two fragments contained the complete gene. The gene contains a single long open reading frame capable of coding for a protein of 1,296 amino acids with a calculated molecular mass of 139.7 kD flanked by consensus promoter sequences, the ribosome binding site, and terminator sequences (18, 19) (Fig. 2). The first 33 amino acids of the putative gene product resemble bacterial signal peptides (20) suggesting that the protein is exported by a sec-dependent mechanism. From position 34–56 the sequence is identical to the 23 amino acids determined by NH_2-terminal sequencing of the purified cytotoxin.

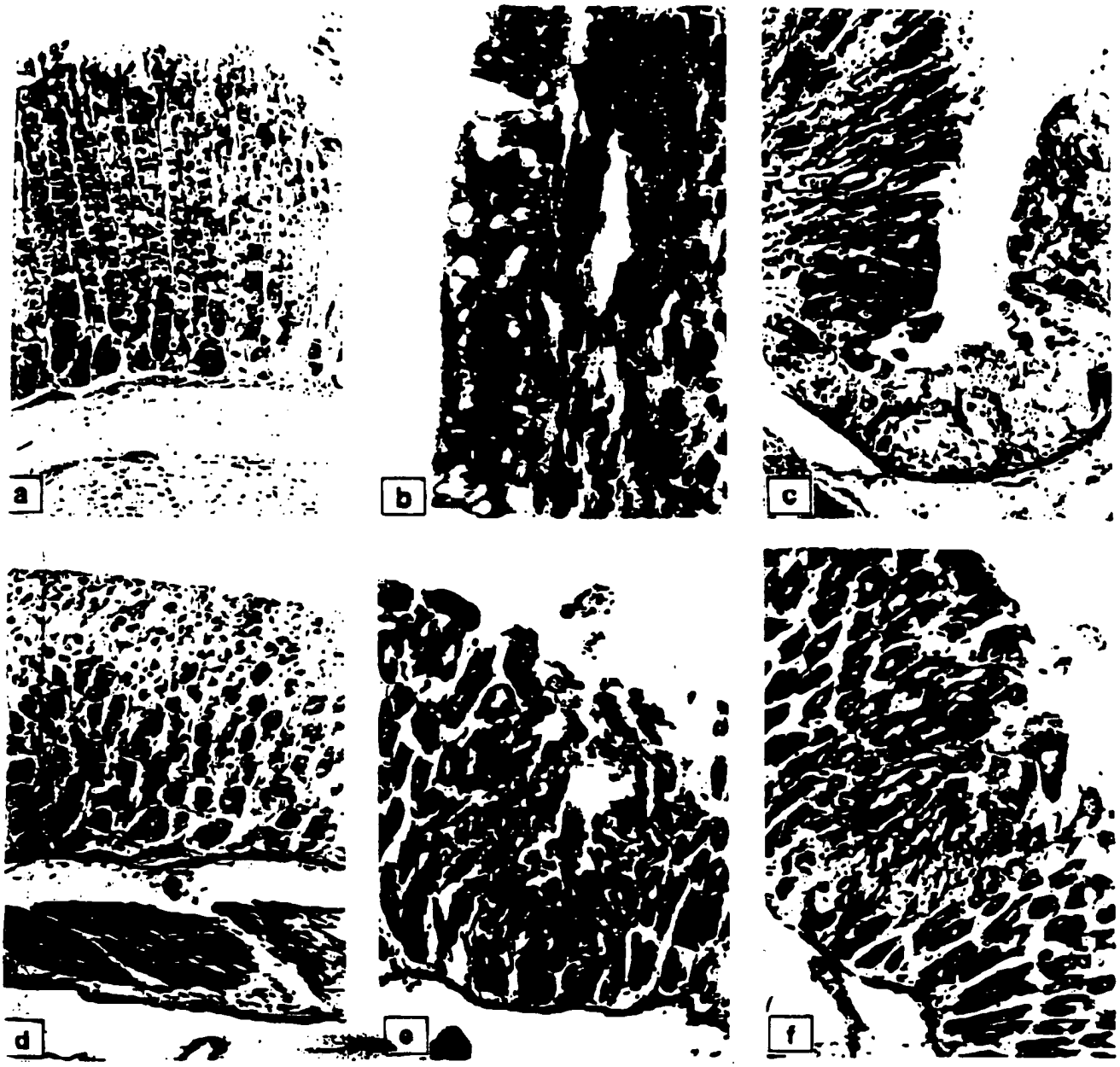

Figure 1. Cell vacuolation and damage to the gastric mucosa induced by *H. pylori* cytotoxin. (*a*) Gastric mucosa of a control mouse treated with saline (200×). (*b* and *c*) Gastric mucosa of a mouse treated with *H. pylori* sonicated (1,000× and 200×). (*d*, *e* and *f*) Gastric mucosa of a mouse treated with purified cytotoxin (200×).

Characterization of the Cytotoxin Gene Products. Different regions of the gene were expressed in *E. coli* as fusion proteins and used to obtain rabbit antisera (fragments A–E in Fig. 3 *a*). Antisera against peptides A, B, and C (Fig. 3 *a*) recognized a 94-kD polypeptide in both cell extracts and culture supernatant (Fig. 3 *b*, lanes *1–3* and *6–8*) of *H. pylori*. Antisera against the COOH-terminal region (peptides D and E in Fig. 3 *a*), failed to recognize this protein, however in the cell extracts they recognized a polypeptide of ~33 kD (Fig. 3 *b*, lanes *9* and *10*). This fragment was barely detectable in the supernatant fraction (Fig. 3 *b*, lanes *4* and *5*) suggesting that the 94-kD active toxin is released from the cell after cleavage of the COOH-terminal fragment of the precursor.

In the culture supernatant, the 94-kD polypeptide was further processed to produce a 37-kD NH_2-terminal fragment

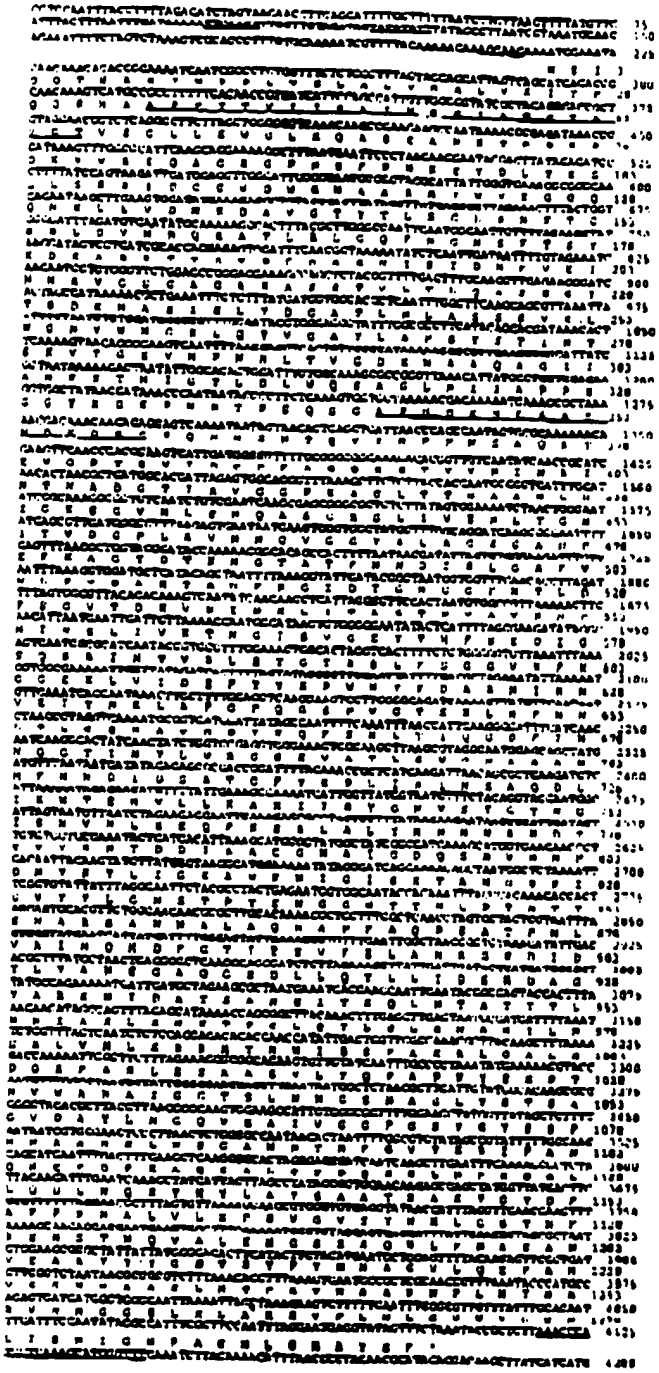

Figure 2. Nucleotide sequence and deduced amino acid sequence of the cytotoxin gene. Putative promoter elements, ribosome binding site, and transcription terminator are underlined. The 23 NH_2-terminal amino acids previously determined and the repeated hydrophilic motif are also underlined.

and a 58-kD COOH-terminal fragment (Fig. 3 *b*, lanes *1–3*). These two polypeptides copurified on cationic exchange chromatography with the 95-kD molecule suggesting that the fragments remain associated after cleavage. NH_2-terminal sequencing of the gel-purified 58-kD fragment revealed that

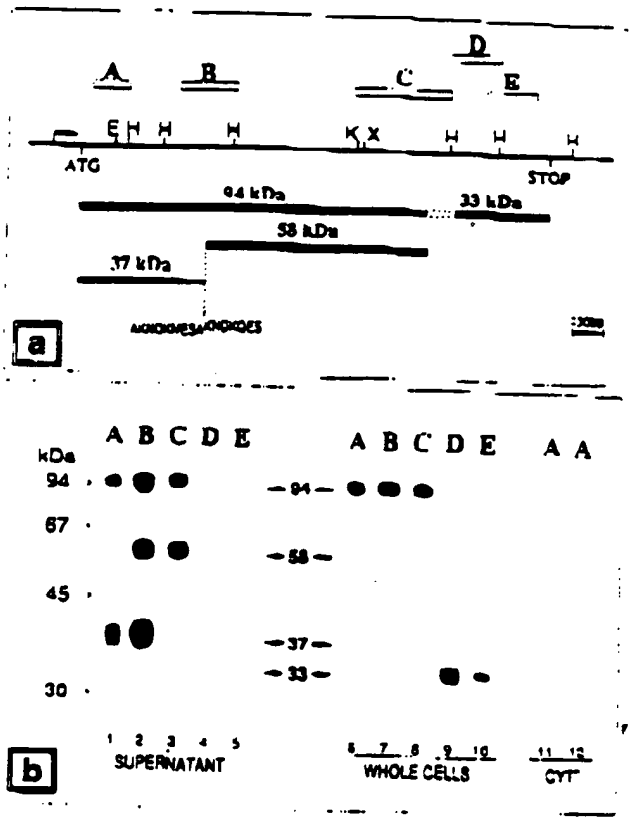

Figure 3. Immunoblot analysis of cell extracts and culture supernatants. (*a*) Schematic map of the fusion proteins used for generating the antisera and the polypeptides detected. (*b*) Lanes *1–5*, Immunoblots of cell extracts or lanes *6–10* of culture supernatants from *H. pylori* strain CCUG 17874 probed with antisera against region A (lanes *1* and *6*), region B (lanes *2* and *7*), region C (lanes *3* and *8*), region D (lanes *4* and *9*), or region E (lanes *5* and *10*). Lane *11*, cell extracts and lane *12* supernatant from a cytotoxin negative strain (G21) probed with antisera against region A.

this processing occurs after the alanine at position 352. This residue is contained in a very hydrophilic region that includes a short almost perfect repeat of the sequence AKNDKXES. Cleavage occurs between the alanine and the lysine of the second repeat. Secondary structure predictions indicate that this region forms a flexible exposed loop in the protein.

Noncytotoxic Strains Contain a Silent Cytotoxin Gene. The antisera failed to detect the cytotoxin in either the cell extracts or culture supernatant of cytotoxin negative strains (Fig. 3 *b*, lanes *11* and *12*). The structure of the cytotoxin gene in three cytotoxin positive and three cytotoxin negative strains was analyzed by PCR using a panel of seven pairs of oligonucleotide primers spanning the complete gene. All pairs of primers used resulted in DNA fragments of the same length as measured by agarose gel electrophoresis, indicating no major rearrangement of the gene (data not shown). This is in contrast to the *cagA* gene which is completely absent in cytotoxin negative strains. We conclude that the cytotoxin gene is present also in noncytotoxic strains but it is not expressed. The reason

for the lack of expression of the gene and its relationship with the *cagA* gene will require further study.

Discussion

We have described a model that reproduces in mice prominent aspects of the pathology associated with *H. pylori* infection in humans. Gastric lesions were found after administration of sonic extracts and the purified cytotoxin from *H. pylori*. The lesions observed after administration of sonic extract reproduced several aspects of the histological lesions observed in biopsies from patients suffering from gastric ulcer disease, including epithelial vacuolation, mucosal erosion, necrosis, and ulceration. Sonicate-induced lesions lacked the neutrophil infiltration of the epithelium that is characteristic of *H. pylori*–associated gastritis in humans, suggesting that live bacteria may cause additional inflammation that the extracts fail to induce. It cannot be excluded however that there may be species differences in the inflammatory response.

The lesions induced by the purified cytotoxin were less severe than th se obtained with the sonic extract, however they clearly showed extensive tissue damage and mucosal erosion. In particular, little if any inflammatory cell infiltration of the lamina propria was observed. A positive relationship between erosion and intraepithelial granulocytes has been observed in *H. pylori*–induced gastritis (21), however, the topography of the granulocytes did not necessarily overlap that of the epithelial er sions. It is likely, therefore, that the toxin induced erosi n is due to direct cytotoxicity rather than an indirect inflammatory mechanism.

The sonicate induced larger, and more abundant vacuoles than the purified cytotoxin. In vitro, the presence of ammonia increases the size and number of toxin induced vacuoles (22). In vivo, the presence of the powerful *H. pylori* urease provides the ammonia that may account for the enlarged vacuoles. At this stage, we cannot exclude the possibility that additional molecules present in the sonicate may contribute to worsening of the lesions initiated by the cytotoxin. In particular, bacterial lipopolysaccharide may contribute to the increased inflammatory cell infiltration of the lamina propria. However the observation that extracts from noncytotoxic strains are unable to induce epithelial lesions in our model clearly indicates that the cytotoxin is necessary to induce the gastric damage. Since the toxin alone is able to induce in mice the mucosal erosion observed in human gastritis, we believe we have fulfilled Koch's postulates in a molecular fashion for this particular aspect of the disease.

The model we have described has several advantages over the existing models of *H. pylori* infection because it overcomes the inability of *H. pylori* to colonize small laboratory animals and the necessity to use surrogate Helicobacter species such as *H. felis*. In addition, pathology is induced in a period of time that is very short compared to the long lasting experiments involving gnotobiotic piglets and monkeys.

The availability of this mouse model of disease combined with the knowledge of the structure of the cytotoxin gene and protein represent the basic tools for understanding the molecular mechanisms of *H. pylori*–induced disease leading the way to the development of therapeutics and vaccines to combat such an important human pathogen.

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Animal models for gastric *Helicobacter* immunology and vaccine studies

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Abstract

Over the last decade animal models have been used extensively to investigate disease processes and therapy for *Helicobacter pylori* infections. The *H. pylori* animal models which have been used in pathogenesis and vaccine studies include the gnotobiotic pig, non-human primates, cats, dogs, and several species of rodents including mice, rats, gerbils and guinea pigs. *H. felis* infection of mice and *H. mustelae* infection of ferrets have also been used. Recently, investigators have begun using transgenic mice and gene-targeted 'knock-out' mice to investigate *Helicobacter* infections. Each of these animal models has distinct advantages and disadvantages which are discussed in this minireview. The choice of an animal model is dictated by factors such as cost and an understanding of how each model will or will not allow fulfillment of experimental objectives. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Helicobacter pylori* vaccine; Transgenic mouse; Gene-targeted knock-out mouse; Gnotobiotic pig; *Helicobacter felis*; *Helicobacter mustelae*; Ferret

1. Introduction

Helicobacter animal models have been used to examine (1) antimicrobial therapies, (2) the oncogenic potential of *Helicobacter* infections, (3) *Helicobacter* virulence factors, (4) host responses to *Helicobacter* infections and (5) *Helicobacter* vaccine development. This minireview will focus on gastric *Helicobacters* which can be propagated in culture and which have been used to study host responses and vaccine development. For a more extensive discussion of other *Helicobacter* species and their use in both host responses and non-immunologic research, the reader is referred to the excellent recent reviews by Fox and Lee [1] and by Eaton [2].

For purposes of this minireview *Helicobacter* animal models can be subdivided into *H. pylori* animal models and animal models utilizing other gastric *Helicobacters* (Table 1). Of the *H. pylori* animal models, non-human primate models are ultimately the most similar to humans but are also the most expensive animal models and are also beset with ethical and endangered species issues. The use of non-human primate *H. pylori* models is also discussed elsewhere in this volume by Dubois.

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Table 1
Helicobacter animal models

I.	*H. pylori* in animals
	Non-human primates
	Gnotobiotic pigs
	Cats, dogs
	Gerbils and guinea pigs
	Mice and rats
II	Other gastric *Helicobacters*
	H. felis in cats, mice, rats
	H. mustelae in ferrets

2. *H. pylori* animal models

2.1. Gnotobiotic pigs

Perhaps the first *H. pylori* animal model to be described was the gnotobiotic piglet. This animal model has been extremely useful in helping to demonstrate that putative *H. pylori* virulence factors such as urease and motility are required for productive infection [3,4]. Although barrier-born pigs have also been infected with *H. pylori* and germ-free piglets can be conventionalized after infection, utilization of this model does require a specialized facility where the piglets can either be derived by cesarean section or, at a minimum, be delivered and maintained under barrier conditions. In addition, while infected piglets have been followed for up to 120 days, they grow quite rapidly and it becomes impractical to continue to house them much beyond 60 days. The character of inflammation induced after infection of piglets differs from human infection in that while lymphocytes/plasma cells are present in both, in contrast to adult humans, infected piglets generally exhibit few neutrophils. In this sense, the *H. pylori*-infected piglet might be a good model for *H. pylori*-infected pediatric patients who also exhibit few neutrophils. Both gross and microscopic ulcers have also been reported in infected piglets, adding further credence to this model [5]. One published vaccine study with *H. pylori* and piglets utilized either oral immunization in the absence of an adjuvant or parenteral immunization and reported only modest reductions in bacterial load but increased pathology (including neutrophils) in immunized, challenged piglets [6]. Despite the fact that the *H. pylori* infection of piglets is a relatively good disease model, it has not been widely used in immunology/vaccine studies, probably in part due to its cost and the need for specialized facilities.

2.2. Dogs and cats

A single report of *H. pylori* infection of gnotobiotic beagle dog pups has been published [7]. Although the pathology observed in infected pups closely reproduced human pathology, the bacterial load appeared to be low and no overt disease or symptoms were noted. The *H. pylori* dog model has not been used in immunology or vaccine studies. Natural or experimental *H. pylori* infection of domestic cats has also been reported [8,9]. Infected cats developed a lymphofollicular gastritis with small to moderate numbers of eosinophils and (rarely) a moderate antral infiltration of neutrophils. It has been suggested that domestic cats might serve as a reservoir for *H. pylori* infection of humans, but this is unlikely. The initial description of the cat *H. pylori* infection was from a closed colony of specific pathogen-free animals and it seems more likely that the infection was transmitted to these cats from a human animal caretaker than the other way round. A single small cat vaccination study has been published. In this study, a 20-fold reduction in bacterial density was reported for immunized and challenged cats when compared to non-immune animals [10]. Thus, the *H. pylori* cat model may be used more widely in the future to study the immune response to *Helicobacter* infections. However, the widespread prevalence of spiral bacteria (see for example, *H. felis* below) in the gastrointestinal tracts of domestic cats limits the usefulness of this model to specific pathogen-free, purpose-bred cats which can be quite expensive. In addition, experimental *H. pylori* infection of cats in our laboratory (unpublished) has yielded only low-grade infections with very mild inflammation. Thus the infection and disease processes of *H. pylori* infection of cats need further verification by additional laboratories.

2.3. Gerbils and guinea pigs

Several recent reports have indicated that Mongo-

lian gerbils can be infected with *H. pylori* and that significant disease, including gastric ulcers, develops [11–13]. Using the gerbil model, *H. pylori* has also been shown to enhance chemical gastric carcinogenesis [14]. As of this writing, no host response or vaccine studies have been published using the gerbil model, although the excellent disease mimics to human infections make it an attractive candidate. The relative lack of immunologic reagents for the gerbil, compared to those available for mice and rats, may hamper progress with this model, however. *H pylori* infection of guinea pigs has also recently been reported [15] The guinea pig model used the mouse-adapted Sydney strain of *H pylori* (see below) and mild to moderate multifocal antral gastritis was reported. Similar to humans, the dietary requirement of guinea pigs for the antioxidant vitamin C may make this model an especially useful one to study the role of *H pylori* in gastric carcinogenesis. Similar to the gerbil, however, a relative lack of immunologic reagents may limit the usefulness of this model for host response and vaccine studies.

2.4 *H. pylori* mouse models

In the late 1980s and early 1990s many laboratories unsuccessfully attempted to establish mouse models of *H. pylori* infection. In 1991 Karita and colleagues reported that T lymphocyte-deficient nude mice could be productively infected with *H pylori* but that under identical conditions only a transient infection was established in immunocompetent mice [16]. Although nude mice are not very useful to study immune responses, this result paved the way for other investigators and over the past 3 years many laboratories have reported *H. pylori* infection in immunocompetent mice [17–22]. Successful mouse models for *H pylori* infection would be ideal for the study of host immune responses as a large variety of immunologic reagents are available for the mouse. In addition, mice are relatively cheap, and literally thousands of inbred strains of mice as well as transgenic and knock-out animals (see below) are available. The biggest downside to the mouse models which have been described to date are the low degree of inflammation and lack of other disease processes observed. There have been some suggestions that selected inbred strains of mice may experience more severe *H. pylori* disease than other inbred strains of mice [21,22], but this has not been a universal finding and disease severity appears much lower than with the mouse *H felis* model (see below). In addition, not all *H. pylori* isolates will infect mice. There appear to be undefined selections or adaptations of *H. pylori* isolates for growth in the mouse stomach and this could affect the disease (or lack thereof) observed in infected animals. While it has been suggested that the presence of the *cag* pathogenicity island may be required for *H pylori* infection of mice, murine infection with CagA/VacA-negative strains of *H. pylori* has been reported [19] The relative lack of disease in *H pylori*-infected mice makes this model a poor one to study *H. pylori*-associated disease. However, the mouse *H. pylori* models are beginning to be used extensively in vaccine studies where the endpoint is the presence or absence of infection rather than disease.

2.5. Other gastric *Helicobacter* infections used in host response and vaccine studies

In addition to *H pylori*, up to two dozen other *Helicobacter* species which infect other animals, many of which also infect gastric tissue, have been described [1]. Two of these non-*H pylori* gastric *Helicobacters* which have been extensively used to study pathogenesis and vaccine development are *H. felis*, which was originally isolated from cats and has been used to experimentally infect mice, and *H mustelae*, which naturally infects ferrets.

2.6 *H felis* infection of mice

In 1990, before the successful development of *H. pylori* mouse models of infection, Adrian Lee and colleagues reported that germ-free mice could be infected with *H felis* [23]. It was subsequently shown that *H felis* could also infect conventional mice and that the severity of disease varied greatly with the inbred strain of mouse infected. Thus, *H. felis*-infected C57BL/6 mice were shown to develop severe gastritis and atrophy which includes replacement of parietal cells by mucus-secreting cells, while BALB/c mice infected under identical conditions developed only mild disease [21,24]. The *H felis* mouse model was also used in the first successful *Helicobacter* vac-

cine studies, before the development of *H. pylori* mouse models [25,26], and it continues to be used in vaccine studies. In spite of the fact that *H. felis* infection yields more severe disease than *H. pylori* infection of mice does, *H. felis* apparently does not contain the *cag* pathogenicity island, nor does it adhere tightly to gastric epithelial cells. Since both of these traits are accepted virulence factors for *H. pylori*, the ultimate usefulness of the *H. felis* mouse model to study *H. pylori* pathogenesis may be questionable.

2.7. *H. mustelae* infection of ferrets

Virtually all of the commercial colonies of ferrets in the USA and many colonies elsewhere in the world are naturally infected with *H. mustelae*. This gastric *Helicobacter* has been associated with an antral predominant lymphocytic ('chronic') gastritis and peptic ulcers as well as MALT lymphomas in the ferret. It has also been reported that *H. mustelae* possesses a *cagA* gene [27], which in *H. pylori* is a marker for the *cag* pathogenicity island. Similar to *H. pylori* infection of gnotobiotic pigs, the ferret and *H. mustelae* have been used extensively to study bacterial virulence factors. A small number of host response and vaccine studies have been done in this model. Two research groups showed that antimicrobial cure of a natural *H. mustelae* infection does not result in immunity to reinfection [28,29]. Thus successful *Helicobacter* vaccination appears to result in immune responses which are qualitatively different from that induced as a result of infection. In another study in ferrets, an oral vaccine trial using an ineffective mucosal adjuvant was unsuccessful and actually resulted in worse disease including ulcers in infant ferrets [30]. Finally, we have shown that oral therapeutic vaccination of ferrets can clear *H. mustelae* from about 30% of these naturally infected animals and can dramatically reduce the severity of disease in the remaining animals [31]. This result with naturally infected animals is a much more rigorous test of the concept of therapeutic immunization than is clearance of *H. felis* infection from experimentally infected mice.

One advantage which *H. mustelae*-infected ferrets as well as *H. pylori*-infected dogs and cats (see above) and other relatively large animals have over smaller rodents is that they can be repeatedly endoscoped to follow infection status and to retrieve biopsies for histologic analysis. The major shortcoming to the use of the ferret in immunologic studies at present is the paucity of reagents. If and when additional reagents become available the ferret *H. mustelae* model should be an extremely useful one for the study of host response and vaccine development.

2.8. Using inbred, transgenic and knock-out mice to study *Helicobacter* infections

Although mouse *H. pylori* and *H. felis* models are limited by their poor disease mimic of human disease and lack of bacterial virulence factors, respectively, there is such a wealth of inbred, mutant and specially created strains of mice available, that mouse *Helicobacter* models will likely continue to receive much attention in the future. In the following paragraphs, examples will be cited from the literature to illustrate the kinds of studies which can be accomplished using these mouse strains. It is not the purpose of this minireview to completely summarize the state of the art of vaccine development or host responses towards *Helicobacter* infections but rather to point out what kinds of studies are possible using these mouse strains. One possible explanation for the low level of pathology observed using most mouse *H. pylori* models is the lack of adherence of *H. pylori* to mouse tissues. This is one of many areas which it may be possible to study using genetically engineered mice. One putative receptor for *H. pylori* involves fucose residues on complex human gastric cell surface polysaccharides. A transgenic mouse has been developed which expresses an enzyme which can add fucose to polysaccharides in mouse gastric tissue. Recent results with this transgenic mouse infected with *H. pylori* have suggested that more severe pathology occurs in the transgenic mouse than in control non-transgenic mice [32]. Additional examples of how the use of inbred and genetically altered mouse strains can advance our knowledge of host responses to *Helicobacter* infections are included in the sections which follow.

2.8.1. *Helicobacter* disease severity is related to variations in host response

Much *Helicobacter* research has focused on viru-

lence factors of the bacteria such as urease, VacA and the *cag* pathogenicity island. As already mentioned above, however, different inbred strains of mice vary in their inflammatory/immune responses to identical *Helicobacter* infections [21,24]. These studies have primarily used the *H. felis* model since the inflammatory response to *H. felis* is much more robust than the inflammatory response to *H. pylori* in the mouse. In addition to the description of these genetic differences, additional mouse strains have been used to try to approach possible mechanisms involved. It has been shown, for example, that whereas lipopolysaccharide (LPS)-responsive C3H/He mice developed severe atrophy after *H. felis* infection, the congenic LPS non-responsive C3H/Hej strain exhibited no atrophy [33]. Further, use of major histocompatibility complex (MHC) congenic mice showed that non-MHC genes appear to play a major role in control of the magnitude of the inflammatory response to *H. felis* infection [24]. In a more recent study, *H. felis*-infected C57BL/6 mice exhibited enhanced apoptosis and fundic epithelial cell proliferation when compared with infected BALB/c or C3H/Hej mice [34]. In this study enhanced apoptosis was correlated with gastric expression of phospholipase A2, a gene product which has recently been associated with formation of polyps in the colon. Finally, the role which interleukin (IL)-10 (an 'anti-inflammatory' T-helper cell type 2 cytokine) plays in the inflammatory process has been examined using wild-type and IL-10 gene-targeted mice in which the IL-10 gene has been 'knocked out'. In this study *H. felis*-infected IL-10 knock-out mice exhibited a more severe and hyperplastic/atrophic gastritis than did wild-type mice with a functioning IL-10 gene [35] indicating a role for this cytokine in control of the inflammatory response.

2.8.2 *The use of immunodeficient mice to study Helicobacter infections*

The studies summarized above hint that immune responses may contribute to the pathology observed after gastric *Helicobacter* infections. We have used severe combined immunodeficient (SCID) mice in our laboratory to more directly examine this question. Wild-type BALB/c mice and BALB/c mice with the SCID mutation infected with *H. felis* exhibited a low level but equivalent inflammatory response [36]. In contrast, C57BL/6 mice, which normally exhibit high inflammation after *H. felis* infection, demonstrated a much lower inflammatory response when mice with the SCID mutation on the C57BL/6 background were infected (Jump, Czinn and Nedrud, unpublished). These results suggest that the immune response makes an important contribution to the gastritis observed after *Helicobacter* infections. The use of inbred strains of mice where tissues can be freely exchanged among members of the inbred strain has allowed us to confirm this hypothesis. We showed that adoptive transfer of spleen cells or T cell lines from *H. felis* infected immunocompetent C57BL/6 mice into naive recipients exacerbated the gastritis in recipient mice if the recipients were challenged with *H. felis* [37].

Elsewhere in the gastrointestinal tract, many strains of immunodeficient or T cell receptor or cytokine knock-out mice have been demonstrated to be susceptible to inflammatory bowel disease. The fact that non-gastric *Helicobacter* organisms may play a role in these models of inflammatory bowel disease was recently demonstrated when it was shown that either SCID mice or T cell-deficient nude rats developed inflammatory bowel disease when they were infected with *H. hilis*, an intestinal *Helicobacter* [38,39].

2.8.3 *The use of inbred and knock-out mice in vaccine development mechanisms of protection*

Based on knowledge from other mucosal pathogens, the initial assumption in *Helicobacter* vaccine studies was that gastric IgA antibodies probably played a major role in preventing or clearing *Helicobacter* infections. Although many different animal models including mice, ferrets, cats and non-human primates have been used in vaccine studies, only recently has the use of several different types of gene-targeted knock-out mice begun to elucidate probable mechanisms of vaccine-induced resistance to infection. The availability of IgA-deficient mice allowed our laboratory to conclude that IgA antibodies are, in fact, not required for protection after oral vaccination [40]. We found, however, that potentially compensating levels of IgM antibodies, which can also be transported onto mucosal surfaces, were produced in these IgA knock-out mice. More recently we and our colleagues have used μMT B cell-defi-

Table 2
Factors to consider when selecting a *Helicobacter* animal model

	Most Favorable			Least
Cost and availability	mice-rats	ferrets/cats	pigs	primates
Reproduces human disease	primates-pigs-gerbils-ferrets			mouse models
Availability of reagents	mice-primates			ferrets-gerbils

cient mice, which produce no antibodies at all, to show that protection from either *H. felis* or *H. pylori* can occur in the absence of any antibodies [41,42]. These results suggested that T cells may play a role in protection from *Helicobacter* infections in these models and our colleagues showed that mice which are deficient in CD4 T cells are indeed not protected from *H. pylori* infection [42,43]. Furthermore, we and others have used cytokine knock-out mice to show that the T cell cytokines IL-4 and perhaps interferon-γ as well may be required for protection from *Helicobacter* infection [44,45]. As is true for inflammation, a role for T cells in protection from *H. felis* has been confirmed by adoptive transfer of *H. felis* specific T cells into naive recipients in inbred mice [37]. Although these results do not demonstrate exactly how T cells or cytokines mediate protective immunity, they do demonstrate the power of using the emerging technology of gene-targeted knock-out animals to answer questions in *Helicobacter* vaccine development and pathogenesis.

3. Conclusions

As summarized in this minireview and Table 2, there is no 'best' animal model for gastric *H. pylori* infections. Rather, each model has strengths and weaknesses and the choice of an animal model depends on experimental objectives. One area which is sure to receive increasing attention in the future, however, is the use of transgenic and knock-out animals. Although it is possible to derive these animals in many species, the large number of transgenic and knock-out mice currently being developed worldwide will place increased emphasis on mouse *Helicobacter* models.

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Therapeutic Intragastric Vaccination against *Helicobacter pylori* in Mice Eradicates an Otherwise Chronic Infection and Confers Protection against Reinfection

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Chronic infection of the gastroduodenal mucosae by the gram-negative spiral bacterium *Helicobacter pylori* is responsible for chronic active gastritis, peptic ulcers, and gastric cancers such as adenocarcinoma and low-grade gastric B-cell lymphoma. The success of eradication by antibiotic therapy is being rapidly hampered by the increasing occurrence of antibiotic-resistant strains. An attractive alternative approach to combat this infection is represented by the therapeutic use of vaccines. In the present work, we have exploited the mouse model of persistent infection by mouse-adapted *H. pylori* strains that we have developed to assess the feasibility of the therapeutic use of vaccines against infection. We report that an otherwise chronic *H. pylori* infection in mice can be successfully eradicated by intragastric vaccination with *H. pylori* antigens such as recombinant VacA and CagA, which were administered together with a genetically detoxified mutant of the heat-labile enterotoxin of *Escherichia coli* (referred to as LTK63), in which the serine in position 63 was replaced by a lysine. Moreover, we show that therapeutic vaccination confers efficacious protection against reinfection. These results represent strong evidence of the feasibility of therapeutic use of VacA- or CagA-based vaccine formulations against *H. pylori* infection in an animal model and give substantial preclinical support to the application of this kind of approach in human clinical trials.

Infection of the human gastroduodenal mucosae by *Helicobacter pylori* is associated with chronic gastritis, peptic ulcers, and gastric malignancies such as adenocarcinoma and low-grade B-cell lymphoma (2, 34, 35). Eradication of chronic *H. pylori* infection of human mucosae with antibiotic therapy markedly alters the natural history of chronic gastritis, peptic ulcers, nonulcer dyspepsia, and low-grade B-cell gastric lymphoma and reduces the rate of relapse of clinical symptoms (44). Problems such as poor patient compliance and increasing occurrence of strains of *H. pylori* resistant to some of the antimicrobials used (i.e., clarithromycin and metronidazole) represent major drawbacks that may limit the efficacy of chemotherapeutic intervention on a large scale (20, 25, 45). Moreover recent evidence suggests that infections cured with antimicrobial agents in adults do not induce immunity against reinfection (39).

Experiments with animal models suggest that mucosal vaccination with either whole-cell preparations or purified antigens plus appropriate adjuvants may prevent infection (5, 16, 17, 24, 27, 28, 31). Development of efficacious strategies that exploit the therapeutic use of vaccines against an ongoing infection represents an attractive therapeutic alternative to the use of antibiotics. Successful eradication of *Helicobacter felis* infection from mice has been reported previously by others using either lysates (13) or the *H. pylori* recombinant urease (6). More recently Cuenca et al. have also reported a low but significant rate of eradication of natural *Helicobacter mustelae* infection in ferrets by therapeutic vaccination with *H. pylori* urease (10).

We (7, 8, 47) and others (3, 15, 46) have shown that severe symptomatic gastric diseases are associated with gastric colonization by a subset of *H. pylori* strains (called type I strains) expressing a potent vacuolating cytotoxin (VacA), which is cytopathic in vitro to various epithelial cells (22, 37) and causes gastric mucosal damage in vivo to mice (19, 42), and that its toxicity is epidemiologically associated with peptic ulcer (1, 9). Type I strains also bear a 40-kb pathogenicity island (PAI) that codes for several disease-associated virulence factors, including the immunodominant antigen CagA (4, 7, 8, 43).

We have recently developed a mouse model of colonization by *H. pylori* (27). This model, which is at variance with previously described animal models that use *Helicobacter* species that do not express neither VacA or CagA, allows assessment of the potential of these molecules as vaccine candidates. Since no formal evidence has yet been reported regarding the feasibility of the therapeutic use of *H. pylori* antigens as vaccines against infection with *H. pylori*, with the mouse model of persistent *H. pylori* infection we have assessed the potential use of antigen formulations that we have already shown to be effective in preventive vaccination as therapeutic vaccines (27, 28).

We report here for the first time, with an animal model of *H. pylori* infection, that an otherwise chronic infection in mice by an *H. pylori* type I strain can be successfully eradicated by intragastric therapeutic vaccination with *H. pylori* antigens (recombinant VacA or CagA) together with a nontoxic mucosal adjuvant. Moreover, we show that cured mice are resistant to reinfection.

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MATERIALS AND METHODS

Preparation of *H. pylori* antigens and LTK63 mutant. *H. pylori* SPM326 was used to prepare the bacterial lysate as previously described (27). Briefly, bacteria cultured as described below were harvested from the plates and suspended in sterile saline; the suspension mixture was then pulse sonicated (Branson Ultra-

sonic, Danbury, Conn.) for 3 min at 50% capacity while kept in an ice bath. The protein concentrations of bacterial lysate were determined with the Bradford reagent (Bio-Rad). Aliquots of the lysate were then snap-frozen in liquid nitrogen and kept at −80°C until used. This lysate was used for both immunizations and enzyme-linked immunosorbent assay (ELISA) plates. Whole recombinant VacA molecule (TOX100) was expressed in *Escherichia coli* and purified by affinity chromatography as described elsewhere (26). We have reported previously (26) that this recombinant form of VacA is nontoxic. Recombinant native CagA antigen was expressed in *E. coli* and purified as described elsewhere (8, 28). The degrees of purity of these *H. pylori* antigens were assessed by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the identity of the purified molecule was confirmed by Western blot analysis with specific antibodies. The LTK63 mutant of *E. coli* labile toxin (LT; bearing an Ser→Lys substitution in position 63) was obtained by site-directed mutagenesis and was purified from the periplasm of a recombinant *E. coli* strain as previously described (36).

Mouse-adapted *H. pylori* strains. The bacterium used for infection was the type I strain SPM326. This strain was adapted to colonize the gastric mucosae of the mouse stomach by repeated in vivo passages consisting of several isolation-reinoculation cycles as previously described (27). Bacteria, cultured in microaerobic conditions as described below, were harvested immediately before inoculations to mice with a sterile cotton swab and resuspended in a small volume of sterile saline. After determination of the optical density at 530 nm, bacterial cell suspensions were diluted to allow intragastric inoculation of 10^9 CFU/0.1 ml to each mouse and were kept on ice until use.

Treatments of mice. Mice were infected as previously described, with slight modifications (27). Briefly, 5-week-old CD1/SPF male mice (Charles River, Calco, Italy) at days 0, 2, and 4, after neutralization of gastric acidity with Na bicarbonate, were challenged intragastrically with *H. pylori* SPM326. The mice were sacrificed at various times after the bacterial inoculation. Colonization of gastric mucosae was assessed as previously described (27, 28). In brief, the mucosal surface of each stomach was gently streaked onto the surface of a separate Columbia agar plate containing 10% horse blood plus amphotericin B (50 μg/ml), vancomicin (100 μg/ml), polymixin B (3.3 μg/ml), bacitracin (200 μg/ml), and nalidixic acid (10.7 μg/ml) (30). The plates were then incubated under microaerobic conditions with the Anaerojar system with the Campygen atmosphere generating system (Oxoid, Basingstoke, United Kingdom) for 4 to 7 days. Growing *H. pylori* colonies were identified by morphology and confirmed by positive rapid urease reaction. Mice were considered not infected when no *H. pylori* colony on the plate on which the stomach was cultured was detected. For the assessment of the efficacy of therapeutic vaccination, mice infected for 6 weeks were treated intragastrically with three weekly doses of saline alone (control) or saline containing either (i) 10 μg of LTK63 mutant alone or (ii) 100 μg of *H. pylori* antigens (TOX100, CagA, or lysate) plus 10 μg of LTK63. At the times indicated, the mice were sacrificed by cervical dislocation, their stomachs were collected, and colonization was determined by culture as described. Data are expressed as percentages of protection. The significance of the differences observed between groups was assessed by Fisher's exact test.

Histology and SEM. Specimens of gastric tissue from infected or noninfected (control) mice were fixed in 4% buffered formalin and processed for histological staining with hematoxylin and eosin as described previously (19). For scanning electron microscopy (SEM) analysis, pieces of infected stomachs were fixed in 3% buffered glutaraldehyde for 20 min. After dehydration in ethanol, the samples were mounted on stubs, coated with gold, and examined at 7.2 kV under a Philips 501 scanning electron microscope (Philips, Eindhoven, The Netherlands).

Titration of *H. pylori* antigen-specific antibodies by ELISA. Briefly, detection of serum antibody titers against *H. pylori* was achieved by ELISAs on 96-well plates coated with whole SPM326 lysate (10 μg/well) or with purified native VacA (42) (0.2 μg/well). Coated wells were blocked with PBS containing 2.7% polyvinylpyrrolidone (Sigma, St. Louis, Mo.). Sera from infected mice were pooled and tested as follows. Serial dilutions of serum samples were incubated at 37°C for 2 h and then washed with PBS. Antigen-specific immunoglobulin G (IgG) titers were determined with affinity-purified, γ-chain-specific, biotin-conjugated rabbit polyclonal antibody (Sigma). Serum *H. pylori* antigen-specific IgA, IgG1, and IgG2a titers were determined with affinity-purified α- or γ1- or γ2a-chain-specific, biotin-conjugated rabbit polyclonal antibodies (Southern Biotechnology Association, Birmingham, Ala.) for 2 h at 37°C. Horseradish peroxidase-conjugated streptavidin (Sigma) was then added to the washed plates, and the plates were incubated at 37°C for 2 h. Antigen-bound antibodies were revealed by the addition of *o*-phenylenediamine as a substrate (Sigma). Antibody titers were determined as previously described (12).

RESULTS

Persistence of *H. pylori* infection in mice. We have previously reported that *H. pylori* strains freshly isolated from patient biopsies and adapted to mouse gastric mucosae by several in vivo passages can establish colonization in mice (27). Infection by type I strains causes epithelial damage and inflammation that becomes evident after 8 to 12 weeks of infecti n. These findings have been subsequently confirmed by others (23, 38). In order to assess the persistence of *H. pylori* infection and to monitor the evolution of the disease induced by the infection, a large group of mice was inoculated intragastrically with the mouse-adapted type I strain SPM326 and monitored for as long as 1 year. At various intervals, groups of mice were sacrificed and gastric colonization was assessed by culture. As shown in Table 1, inoculation of mice with an adapted strain of *H. pylori* results in the colonization of their gastric mucosae that is persistent for as long as 1 year. The numbers of colonies that were recovered from the stomachs were also stable over the examined period of time and varied between 10^3 and 2×10^4 in the majority of the infected mice (not shown). The pathological changes induced by infection were followed by histological examination. Figure 1 shows pictures of representative histological fields of gastric mucosae of uninfected control mice (Fig. 1A) and of mice sacrificed at different times after the onset of infection (Fig. 1B through F). At 8 weeks of infection, in agreement with our previously published data (27), gastric lesions consisted mainly of focal epithelial damage. Fields showing epithelial erosive-reparative lesions with polymorphonuclear leukocytes infiltrating the lesions and the surrounding mucosae were present in most of the infected mice (Fig. 1B). However, these lesions were less frequent in the mice observed at subsequent times. Figure 1G is an SEM micrograph obtained from a mouse infected for 8 weeks showing an *H. pylori* cell adhering to the gastric epithelial surface. At 16 and 34 weeks of infection, the most frequently observed lesions consisted of inflammatory cells (mainly mononuclear cells) infiltrating the lamina propria in the superficial layers of the mucosae (chronic superficial gastritis) (Fig. 1C and D). Polymorphonuclear cells were also found to be mainly associated with the surface epithelial layer (Fig. 1D). At 34 weeks of infection, small lymphoid aggregates were also found in the laminae propriae at the base of the glands (Fig. 1E). In mice infected for 1 year or longer, the main histopathological finding consisted in a follicular gastritis, with large lymphoid follicles present in the mucosae as well as in the submucosa (Fig. 1F).

TABLE 1. Persistence of infection by the type I strain SPM326[a]

Time (wk)	No. of mice		% of infection
	Group total	Infected	
1	19	17	89
2	25	23	92
4	27	27	100
6	40	39	98
8	20	19	95
16	18	17	94
34	11	11	100
52	18	16	89

[a] CD1/SPF mice (n = 178) were infected with three intragastric challenges of 10^9 CFU of *H. pylori* type I strain SPM326 as described in Materials and Methods. At different time intervals, groups of mice were sacrificed and the extent of gastric colonization was assessed.

The serum antibody response during infection was monitored by ELISA with *H. pylori* antigens. Figure 2A and C shows that with an *H. pylori* whole-cell sonicate or purified native VacA as solid-phase antigens in the ELISA, IgG and IgA titers were already detectable at 4 to 8 weeks after the onset of infection and increased slowly with time. The IgG response was further assessed by determining the patterns of IgG1 and IgG2a isotypes during infection. Figure 2B and C shows that

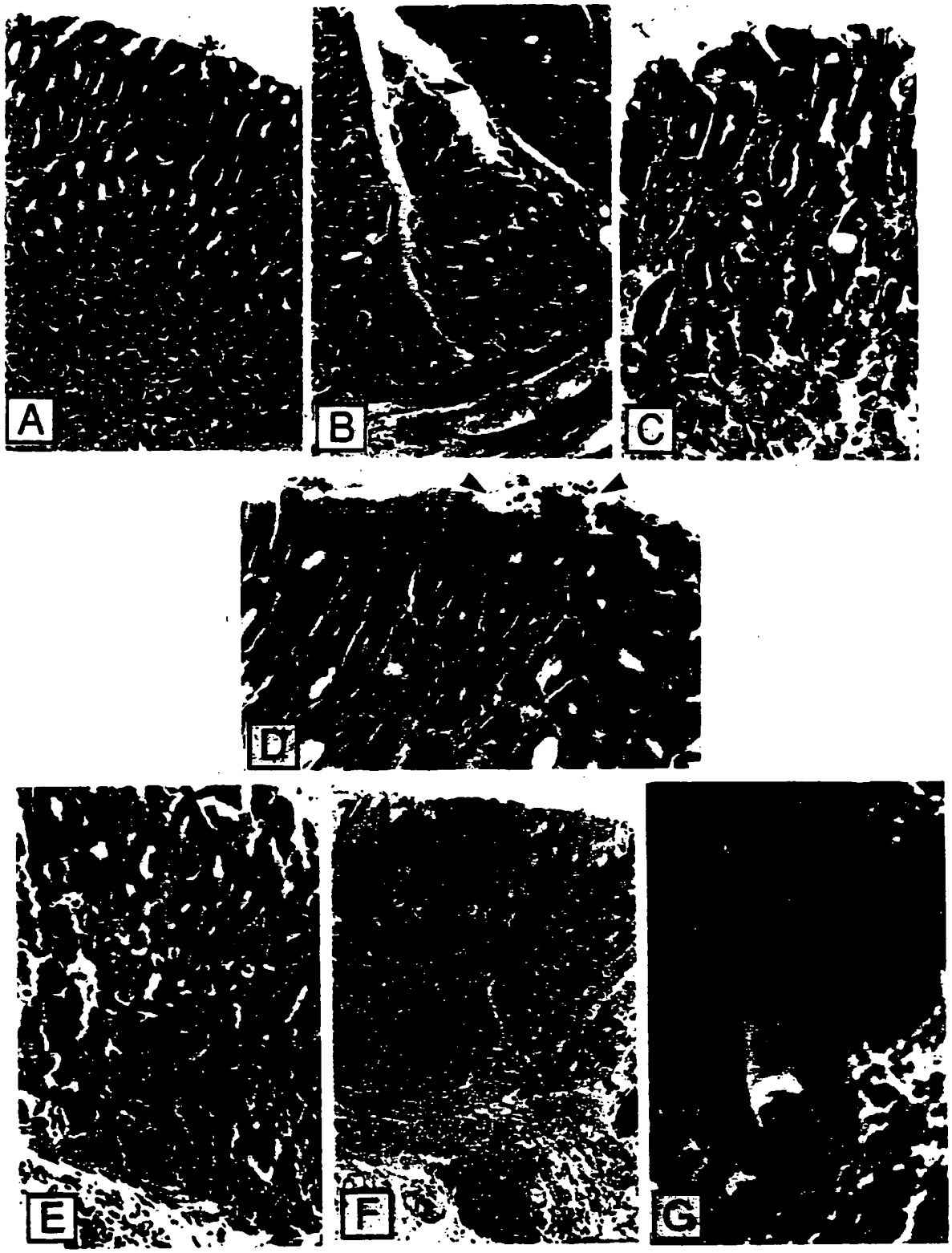

FIG. 1. Histopathology of infected mice. (A) Oxyntic mucosae of a control mouse (original magnification, ×200; (B) oxyntic mucosae of a mouse infected for 2 months showing erosive-reparative lesions (arrows) (original magnification, ×200; (C and D [original magnification, ×400]) superficial chronic inflammatory cells in the laminae propriae (arrows) of mice infected for 4 months (neutrophil polymorphs are also present [arrowheads in panel D] on the superficial epithelium and are associated with epithelial degeneration) (E) lymphocytic aggregate at the base of the glands in the oxyntic mucosae of a mouse infected for 8 months; (F) follicular gastritis with large lymphoid follicles present in the oxyntic mucosae and in the submucosae of a mouse infected for 1 year (original magnification, ×200; (G) SEM of the gastric epithelial surface of a mouse infected for 2 months showing an *H. pylori* cell adhering to the gastric cells (original magnification, ×15,000).

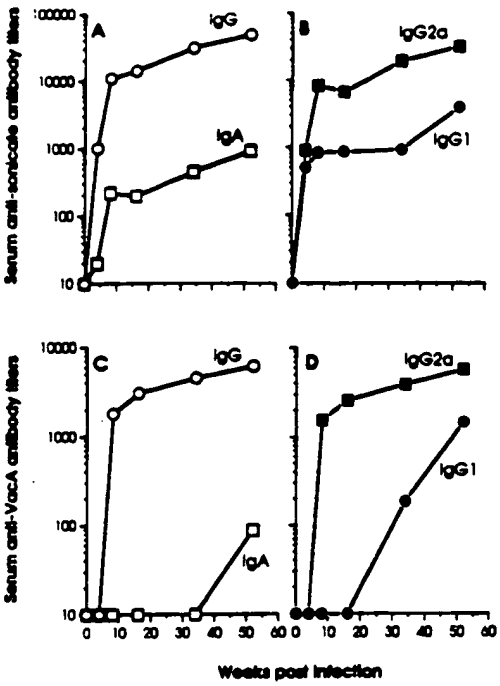

FIG. 2. Serum antibody response during infection. Anti-*H. pylori* antigen-specific titers in pooled sera from CD1/SPF mice during infection with *H. pylori* type I strain SPM326. (A and B) IgG and IgA titers and IgG1 and IgG2a serum titers, respectively, against whole-cell sonicate. (C and D) IgG and IgA titers and IgG1 and IgG2a serum titers against VacA, respectively.

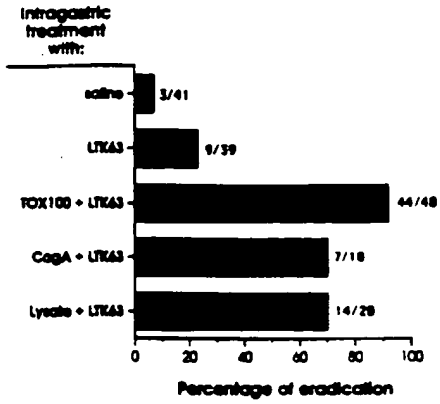

FIG. 3. Therapeutic intragastric vaccination: cumulative results of five experiments. Mice that had been infected 6 weeks previously with *H. pylori* type I strain SPM326 received three weekly intragastric treatments with the indicated vaccine formulations. Control groups received either saline alone or saline containing the adjuvant LTK63 alone. The statistical significance of the observed differences was assessed by Fisher's exact test as described in Materials and Methods. The percentages of protected (noninfected) mice in groups receiving TOX100 or lysate plus LTK63 were significantly different ($P \leq 0.0001$) compared to groups receiving saline alone or LTK63. Treatment with CagA plus LTK63 also induced highly significant eradication compared to treatment with saline alone ($P \leq 0.0001$) or with LTK63 alone ($P \leq 0.02$). The level of protection achieved by administration of lysate plus LTK63 was also significantly different from that obtained with either saline alone ($P \leq 0.0001$) or LTK63 alone ($P \leq 0.002$).

IgG2a was the prevailing IgG isotype against both the whole-cell sonicate and VacA.

Therapeutic vaccination eradicates infection. To assess the feasibility of therapeutic vaccination, mice were infected with *H. pylori* SPM326 and, 6 weeks later, received three weekly doses of 100 μg of the recombinant VacA (TOX100), CagA, or a whole SPM326 lysate together with 10 μg of the mucosal adjuvant LTK63. Control groups received either saline alone or saline containing LTK63. All mice were sacrificed 1 weeks aft r the last intragastric treatment, and gastric colonization by *H. pylori* was assessed by culture. Figure 3 shows the cumulative results of five experiments. Highly significant levels of eradication were achieved when infected mice were therapeutically vaccinated with TOX100, or CagA, or whole-cell lysates administered intragastrically together with LTK63 (for all vaccine groups, $P \leq 0.0001$ versus saline-treated mice), while treatment with adjuvant alone induced a slight but not statistically significant eradication. To better assess the effect of therapeutic vaccination, the numbers of colonies recovered from infected mice that received the treatments were determined. Table 2 shows the cumulative results of two experiments in which the mice were therapeutically vaccinated with TOX100 or with CagA. In the small proportion of mice in which infection resulted after the therapeutic vaccination, a marked decrease in the relative amount of *H. pylori* colonies recoverable from their stomachs was observed, compared to that for mice which received control treatments (i.e., LTK63 alone or saline).

In order to assess whether the observed eradication persisted with time, 80 mice were infected with *H. pylori*. Six weeks later, half of them were treated intragastrically with three weekly doses of 100 μg of TOX100 together with the adjuvant LTK63. The remaining 40 mice received LTK63 al ne. Groups of 10 mice in the control and in the vaccine groups were sacrificed at different times after the intragastric treatment, and gastric colonization by *H. pylori* was assessed. Figure 4 shows that eradication achieved with intragastric therapeutic vaccination was stable and that as many as 70% of the mice that were treated intragastrically with TOX100 plus the adjuvant LTK63 remained noninfected for at least 3 months after therapeutic vaccination.

Therapeutic vaccination confers protection against reinfection. Then, we asked whether mice in which infection had been eradicated by therapeutic vaccination were protected from reinfection. Mice were infected with strain SPM326 as described above and, 6 weeks later, received three weekly intragastric treatments with LTK63 alone (control) or with LTK63 together with TOX100 (vaccine). A group of mice from both th control and vaccine groups were sacrificed 1 week after the last intragastric treatment, and gastric colonization by *H. pylori* was assessed by culture. Figure 5A shows that as expected from data shown above, 80% of the mice that received the thera-

TABLE 2. Number of *H. pylori* colonies recovered in plates from infected-vaccinated mice[a]

Treatment	No. of mice		No. of mice with the following no. of colonies:		
	Total	Infected	1–100	101–1,000	>1,000
Saline	18	17		2	15
LTK63	8	6			6
TOX100 + LTK63 or CagA + LTK63	28	5	3	1	1

[a] Mice were infected with an *H. pylori* type I strain and, 6 weeks later, received three weekly doses of intragastric therapeutic vaccination with TOX100 or CagA plus the adjuvant LTK63. Control groups consisted of mice that received either saline alone or the LTK63 adjuvant alone. One week after the final treatment, the mice were sacrificed and the extent of gastric infection by *H. pylori* was assessed as described. *H. pylori* colonies were detected by visual inspection of the plates and were counted. Data pooled from two separate experiments are shown.

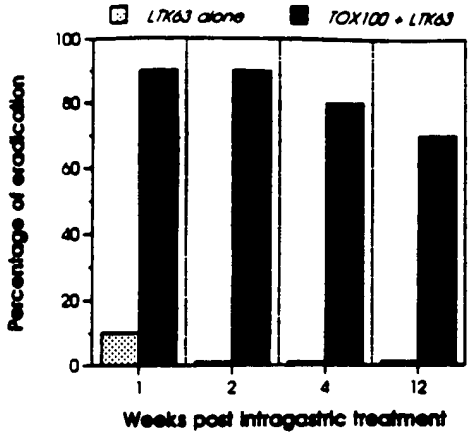

FIG. 4. Persistence of *H. pylori* eradication induced by therapeutic vaccination. A group of 80 mice was infected with strain SPM326. Six weeks later, half of them received three weekly intragastric treatments with TOX100 plus LTK63 (vaccine), while the remaining mice received LTK63 alone (control). At 1, 2, 4, and 12 weeks after the last treatment, 10 mice per group were sacrificed to assess colonization as described in Materials and Methods. Eradication in mice receiving the vaccine was significantly higher than that in mice receiving control treatment at 1 week ($P \leq 0.003$), 2 weeks ($P \leq 0.0001$), 4 weeks ($P \leq 0.002$), and 12 weeks ($P \leq 0.01$) after intragastric treatments. On the other hand, no statistically significant difference was observed among groups of mice that received TOX100 plus LTK63 and that were sacrificed at different times after treatment.

peutic intragastric vaccination were cured. At the same time, the remaining mice from both the control and vaccine groups were rechallenged intragastrically with three doses of 10^9 CFU of strain SPM326 per mouse over a week. Two months after the rechallenge, the mice were sacrificed to assess gastric infection by *H. pylori*. Figure 5B shows that as many as 70% of the mice that were cured by intragastric therapeutic vaccination with TOX100 plus LTK63 were resistant to a subsequent challenge with *H. pylori*. These results clearly show that therapeutic vaccination not only successfully eradicates an otherwise chronic infection but also prevents a subsequent reinfection with *H. pylori* in the majority of vaccinated animals.

DISCUSSION

The results shown in this paper demonstrate that an otherwise chronic gastric infection of *H. pylori* in mice can be successfully cured with oral administration of either an *H. pylori* sonicate or purified recombinant nontoxic VacA or CagA plus a nontoxic mutant of LT as an adjuvant. It has been reported that oral administration of either sonicates or purified recombinant urease B subunit plus cholera toxin (CT) eradicates infection in mice previously infected with *H. felis* (6). Use of the mouse *H. felis* model has also allowed establishment of the first evidence of the feasibility of preventive vaccination against a chronic gastric infection by a *Helicobacter* species (5). However, *H. felis* is not a human pathogen and, more importantly, does not express some *H. pylori* pathogenic determinants (i.e., VacA and antigens encoded by the *cag* PAI, including CagA) that are thought to be involved in human disease (47). In fact, the gastric mucosae of symptomatic infected patients most frequently harbor type I *H. pylori* strains that bear the *cag* PAI, which contains several genes involved in pathogenesis, including the immunodominant disease marker product of the gene *cagA* (4, 8). These type I strains also secrete the toxin VacA, which is hypothesized to be involved in the genesis of ulcerative lesions (1, 9, 19, 22, 37, 41, 42). Infection with type I strains is also strongly associated with the increased risk of occurrence of gastric adenocarcinoma (3) and low-grade gastric B-cell lymphoma (15). The use of the mouse model of *H. pylori* infection that we have devel ped has allowed us to support the concept that type I strains have enhanced virulence compared to Type II strains that do not bear the *cag* PAI and do not express cytotoxic activity (4, 27). Therefore, this animal model of infection is a powerful tool to study the pathogenesis of infection and to develop vaccination strategies with, as vaccine candidates, molecules such as VacA and CagA, which are expressed only by type I *H. pylori* strains, play a major role in human disease, and cannot be tested in the *H. felis* model.

In the present paper, we have shown that the type I strain SPM326 can establish a chronic infection in mice that is stable for at least 1 year. The infection elicits evident gastric lesions, i.e., epithelial erosions and infiltration of inflammatory cells in the laminae propriae of the gastric mucosae. Evident chronic gastritis has also been reported for long-term infection of mice with *H. felis* (33); however, in that model, no evidence of overt mucosal erosions has been shown. We have shown here that long-term infection of mice by *H. pylori* also elicits the appearance of lymphoid follicles in the mucosae, a type of lesion that is also frequently present in chronically infected humans and that in some patients precedes the emergence of low-grade gastric B-cell lymphoma (35).

Our results show that infection of CD1 mice with *H. pylori* induces serum IgG and IgA antibodies which persist for the duration of infection (Table 1 and Fig. 2). It is interesting to note that although both IgG1 and IgG2a isotypes are produced, IgG2a antibodies represent the prevailing isotype. This was even more evident when titers of VacA-specific IgG were determined. This result suggests that chronic *H. pylori* infection in CD1 mice is associated with a predominant activation of

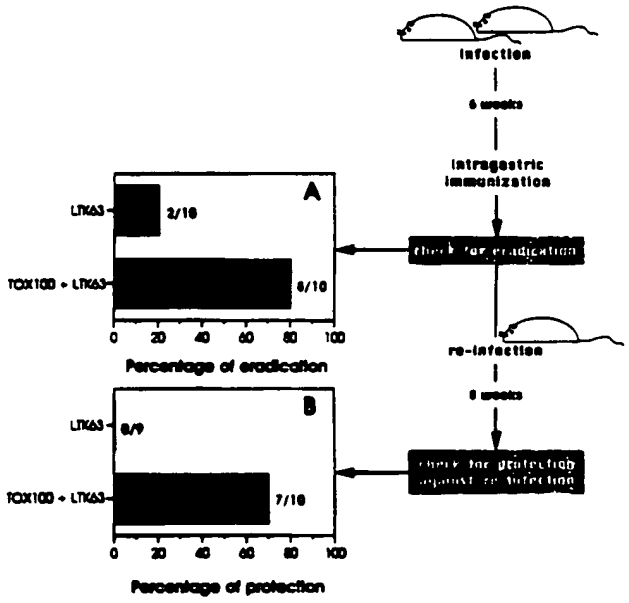

FIG. 5. Therapeutic vaccination confers protection against reinfection. A group of 80 mice were infected with strain SPM326. Six weeks later, half of the mice received three weekly therapeutic vaccinations with TOX100 plus LTK63 (vaccine), while the remaining mice received LTK63 alone (control). (A) At 1 week after the last treatment, 10 mice in the control group and 10 mice in the vaccine groups were sacrificed to assess the level of eradication achieved. (B) The remaining mice were reinfected as described, and 8 weeks later protection was assessed. Both therapeutic eradication and protection against rechallenge obtained in mice that received vaccine treatment were statistically significant ($P \leq 0.03$ and ≤ 0.01, respectively) compared to groups that received control treatment.

Th1-type cell populations. This is in full agreement with previous results obtained by others with a mouse model of infection with *H. felis* (32) and also for humans with *H. pylori*-associated peptic disease (11).

Therapeutic vaccination of *H. pylori*-infected mice with TOX100 or CagA together with the adjuvant LTK63 induces a high level of eradication that persists for at least 12 weeks after the vaccination. The fate of gastric pathology following therapeutic vaccination could not be optimally assessed after eradication, since in most of the experiments the mice were vaccinated at 6 weeks of infection, i.e., when the gastric pathology was not yet fully evident in all mice. Furthermore, in these experiments the mice were sacrificed 1 week after the end of the treatment. However, in the experiment in which the persistence of eradication was assessed, vaccinated mice that were sacrificed at times later than 1 week did not develop gastric pathology and, in particular, no signs of epithelial erosions could be found compared to infected LTK63-treated controls (not shown).

In the experiments reported in the present paper, we did not observe any major change in the serum antibody responses of infected mice after they were immunized with *H. pylori* antigens in the presence of LTK63 as a mucosal adjuvant (not sh wn). These data are in agreement with those reported with the mouse model of infection with *H. felis*, in which therapeutic immunization with recombinant *H. pylori* urease was studied (6). Considering that as discussed above, chronic *H. pylori* infection seems to be associated with a preferential activation of Th1 cell subpopulations, it is tempting to speculate that immunization procedures leading to a substantial activation of Th0- or Th2-type cell populations at the local (mucosal) and/or systemic level may favor eradication of an otherwise chronic infection with *H. pylori*. This hypothesis is sustained by recent experimental evidence obtained both in vivo (17) and in vitro (21). In our experimental procedure, this may have been mediated by the mucosal adjuvant, the nontoxic LT mutant LTK63. Previous work (14, 29, 40, 48) has clearly shown that nontoxic mutants of both LT and CT coadministered with antigens at the mucosal level drive preferential activation of Th0- or Th2-type $CD4^+$ cells, depending on the experimental model employed. This hypothesis may explain the finding that approximately 20% of the mice receiving the LTK63 adjuvant alone exhibited a nonspecific eradication of *H. pylori* infection. However, this nonspecific effect was transient, since it was not observed when gastric colonization was assessed more than 1 week after the last intragastric treatment with LTK63.

There is no full agreement on whether previous infection eradicated by antibiotic therapy in infected humans could confer immunity against a subsequent reinfection. Although most of the reinfections in eradicated adult patients seem to be explained by a recurrence of the infection with the same strain due to failure in the antibiotic regimen used, a recent report has shown that a previous state of chronic infection by *H. pylori*, which had been successfully eradicated by antibiotic therapy, did not confer immunity against reinfection (39). It has also been recently reported that ongoing chronic infection does not protect from superinfection with a new strain (18). In agreement with previous data obtained by others using the mouse model of infection with *H. felis* and treatment with recombinant urease (29), the results presented in the present paper show that mice cured by therapeutic vaccination with recombinant VacA and CagA are resistant to a challenge with three doses of 10^9 CFU of *H. pylori*. These data dem nstrate that vaccination may play an important role, either alone or in association with currently available antibiotic chemotherapy, in reducing the rate of reinfecti n and/or recrudescence in previously infected individuals.

Taken t gether, our results provide strong evidence that an otherwise experimental chronic infection by a type I *H. pylori* strain, which is more frequently isolated from patients with severe diseases, can be successfully cured by therapeutic vaccination with purified recombinant VacA and CagA coadministered with the nontoxic LT mutant LTK63 as a mucosal adjuvant. Previously, this conclusion could be inferred only by evidence obtained from animal models that employed infections with surrogate *Helicobacter* species, such as *H. felis* and *H. mustelae*, that are not human pathogens and, more importantly, do not express the type I *H. pylori*-specific set of pathogenic determinants (i.e., VacA, CagA, and the other factors encoded by the *cag* PAI) that have a pivotal role in the induction of disease in humans. Finally, these data strongly support the concept of developing therapeutic vaccine formulations based on these antigens for safe use in human clinical trials.

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EXHIBIT H

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(57) Abstract

The present invention relates to a method of protection against or treatment for infection by *H. pylori* comprising non-mucosal administration of an effective amount of one or more *H.pylori* antigens.

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IMMUNIZATION AGAINST AND TREATMENT FOR INFECTION BY *H.PYLORI*

This application is related to United Kingdom patent applications GB 9809398.2 (filed 30th April 1998) and GB 9820976.0 (filed 25th September 1998), which applications are hereby incorporated in their entirety by reference.

Technical field

The present invention relates to a method of protection against or treatment of an *H.pylori* infection, comprising non-mucosal administration of an effective amount of one or more *H.pylori* antigens.

Background of the Invention

H.pylori was isolated in 1982 by B. Marshall and J. Warren using microaerophilic conditions that had been developed to grow *Campylobacter jejuni*. *H.pylori* bacteria are S-shaped, gram negative bacilli 2-3.5 μm in length and 0.5-1 μm in width (Blaser, M.J., and J. Parsonnet. 1994. J. Clin. Invest. 94:4-8). It is now (only recently) known that infection with *H.pylori* is the most common infection in the world. In developing countries 80% of the population is infected by the bacterium at the age of 20, while in developed countries *H.pylori* infection increases with age from <20% in 30-year old people to >50% in 60-year olds (Axon, AT. 1995. Pharmacol. Ther. 9:585-588; Blaser and Parsonnet, 1994). The infection is transmitted by either the oro-fecal or the oro-oral route (Blaser and Parsonnet, 1994). Infection occurs during the first years of life and persists forever. Once established, the infection is chronic, possibly permanent. Risk factors for infection are crowding, poor hygiene and host-specific genetic factors.

The complete genome sequence of *H.pylori* has been published (Tomb, J.-F., *et al.* 1997. Nature 388:539-547). A brief review of this article and a general review of the biology of *H.pylori* can be found in Doolittle, R.F. 1997. Nature 388:515-516.

Chronic infection of the human gastroduodenal mucosae by *H.pylori* is frequently associated with chronic gastritis, peptic ulcer, and increases the risk of occurrence of gastric malignancies such as adenocarcinoma and low grade B cell lymphoma (Blaser and Parsonnet, 1994; Parsonnet, J., *et al.* 1991. N. Engl. J. Med. 325: 1127-1231; Parsonnet, J., *et al.* 1994. N. Engl. J. Med. 330: 1267-1271). Most of the infections remain asymptomatic,

whereas symptomatic, severe diseases correlate epidemiologically with the infection by a subset of *H.pylori* strains, called Type I (Blaser, M.J., *et al.* 1995. Cancer Res. 55:2111-2115; Covacci, A., et al. 1997. Trends Microbiol. 5:205-208; Covacci, A., *et al.* Proc. Natl. Acad. Sci. U.S.A. 90:5791-5795; Eck, M., *et al.* 1997. Gastroenterology. 112:1482-1486; Xiang, Z., *et al.* 1995. Infect. Immun. 63:94-98). This subset of strains is endowed with increased virulence due to the expression of a biologically active toxin (VacA), which is cytopathic to gastric epithelial cells *in vitro* and *in vivo* (Ghiara, P., *et al.* 1995. Infect. Immun. 63:4154-4160; Harris, P.R., *et al.* 1996. Infect. Immun. 64:4867-4871; Telford, J.L., *et al.* 1994. J. Exp. Med. 179:1653-1658), and also to the acquisition of a pathogenicity island (PAI), called *cag*, which contains a set of genes encoding several virulence factors (Censini, S., *et al.* 1996. Proc. Natl. Acad. Sci. USA. 93:14648-14653) which are responsible for the induction of the synthesis of the neutrophil chemotactic cytokine IL-8 by the gastric epithelial cells (Censini *et al.*, 1996).

H.pylori factors that have been identified so far include the flagella that are probably necessary to move across the mucus layer, see for example, Leying *et al.* 1992. Mol. Microbiol. 6:2863-2874; the urease that is necessary to neutralize the acid environment of the stomach and to allow initial colonization, see, for example, Cussac *et al.* 1992. J. Bacteriol. 174:2466-2473, Perez-Perez *et al.* 1992. J. Infect. Immun. 60:3658-3663, Austin, *et al.* 1992. J. Bacteriol. 174:7470-7473, WO 90/04030; the *H.pylori* cytotoxin (sometimes referred to as VacA, as it causes vacuolation), see, for example, WO 93/18150, Telford, J.L. *et al.* 1994. J. Exp. Med. 179:1653-1658, Cover *et al.* 1992. J. Bio. Chem. 267:10570-10575, Cover *et al.* 1992. J. Clin. Invest. 90:913-918, Leunk, 1991. Rev. Infect. Dis. 13:5686-5689; the *H.pylori* heat shock proteins (hsp), see, for example, WO 93/18150, Evans *et al.* 1992. Infect. Immun. 60:2125-2127, Dunn *et al.* 1992. Infect. Immun. 60:1946-1951, Austin *et al.*, 1992; and the cytotoxin-associated protein, CagA, see, for example, WO 93/18150, Covacci, A., *et al.* 1993. Proc. Natl. Acad. Sci. USA 90:5791-5795, Tummuru, M.K., *et al.* 1994. Infect. Immun. 61:1799-1809.

Currently, *H.pylori* strains can be partitioned into at least two major groups, which either express (Type I) or do not express (Type II) the cytotoxin (VacA) and the CagA proteins. Type I strains contain the CagA and toxin genes and produce active forms of these antigens. Type II strains lack the CagA locus and fail to express the cytotoxin. The association between the presence of the CagA gene and cytotoxicity suggests that the

production of the CagA gene is necessary for the transcription, folding, export or function of the cytotoxin. Epidemiological analysis indicate that Type I bacteria are associated with duodenal ulcerations, gastric ulceration and severe forms of active gastritis.

For a general review of the pathogenic role of *H.pylori* in peptic ulcer, see Telford, J.L., *et al.* 1994. TIBTECH 12:420-426.

H.pylori culture supernatants have been shown by different authors to contain an antigen with a molecular weight of 120, 128 or 130 kDa (Apel, *et al.*. 1988. Zentralblat für Bakteriol. Microb. Und Hygiene 268:271-276; Crabtree, *et al.* 1992. J. Clin. Pathol. 45:733-734; Cover, *et al.* 1990. Infect. Immun. 58:603-610; Figura, *et al.* 1990. *H.pylori, gastritis and peptic ulcer* (eds. Malfrtheiner, *et al.*), Springer Verlag, Berlin). Whether the difference in size of the antigen described was due to interlaboratory differences in estimating the molecular weight of the same protein, to the size variability of the same antigen, or to actual different proteins was not clear. This protein is very immunogenic in infected humans because specific antibodies are detected in sera of virtually all patients infected with *H.pylori* (Gerstenecker, *et al.* 1992. Eur. J. Clin. Microbiol. 11:595-601).

A protein known as NAP (neutrophil activating protein – Evans D.J., *et al.* 1995. *Gene* 153:123-127; WO 96/01272 & WO 96/01273, especially SEQ ID NO:6; see also WO 97/25429), which is found in both type I and II strains, appears to be protective when tested in the *H.pylori* mouse model (Marchetti, M., *et al.* 1995. Science. 267:1655-1658). NAP is a homodecamer of 15 kDa subunits, and it has been proposed that the multimeric complex has a ring-shaped structure which spontaneously forms hexagonal paracrystalline structures. The assembled protein appears to interact with glycosphingolipid receptors of human neutrophils.

A number of other *H.pylori* antigens are described in WO 98/04702, including ureaseB (SEQ ID NO: 4), HopX (SEQ ID NO: 21), HopY (SEQ ID NO: 21), 36 kDa (SEQ ID NO: 26), 42 kDa (SEQ ID NO: 25), and 17 kDa (SEQ ID NO: 27). Urease is also described in, for example, EP-B-0367644 (protein with urease activity), EP-A-0610322 (ureaseE, F, G, H and I), EP-A-0625053 (urease protein) and EP-A-0831892 (multimeric forms of urease). Other *H. pylori* antigens include the 54 kDa (SEQ ID NO: 2) and 50 kDa (SEQ ID NO: 1) proteins described in EP-A-0793676.

Discussions of various virulence factors of *H.pylori* can be found in, for example, EP-A-93905285.8 and EP-A-96908300.5.

Colonization of the mucosa of the stomach by *H.pylori* is today recognized as the major cause of acute and chronic gastroduodenal pathologies in humans (Blaser and Parsonnet, 1994; Covacci *et al.*, 1997). The recognition of the infectious nature of the illness is having a major impact in the treatment of the disease that is shifting from the treatment of symptoms by anti-H2 blockers to the eradication of the bacterial infection by antibiotic regimen.

In spite of the unquestionable successes that will be achieved with antibiotic treatment, it should be remembered that such treatment inevitably leads to the occurrence of resistant strains that in the long term will make antibiotics ineffective. This suggests that vaccination, which classically is the most effective way to prevent and control infectious diseases in a large population, could be used to prevent infection and possibly also to treat the disease.

The increasing importance of *H.pylori* in the induction of a wide variety of gastric pathologies has represented a major challenge for the development of efficacious prophylactic and/or therapeutic strategies. To better understand the interactions between the bacterium and the host, much effort has focused on the development of appropriate animal models of infection reproducing aspects of the natural human infection.

Several animal models of infection and disease have been developed aiming at studying the pathogenesis of infection and development of preventive and therapeutic strategies. Many of these models are highly impractical, since they employ monkeys (Dubois, A., *et al.* 1994. Gastroenterology. 106:1405-1417) or species that are kept under gnotobiotic (that is, germ-free) conditions, for example germ-free dogs or piglets (Krakowka, S., *et al.* 1987. Infect. Immun. 55: 2789-2796; Radin, M.J., *et al.* 1990. Infect. Immun. 58: 2606-2612). Colonization of gnotobiotic piglets (Krakowka *et al*, 1987) has been reported using *H.pylori* strains isolated from patients with gastroduodenal diseases. However piglets cannot be kept under germ-free conditions for more than 2 months (Radin *et al.*, 1990) mainly due to their nutritional needs.

Successful infection of specific pathogen-free (SPF) cats has been described using a strain isolated from conventional cats (Fox, J.G., *et al.* 1995. Infection and Immunity. 63: 2674-2681; Handt, L.K., *et al.* 1995. J. Clin. Microbiol. 33:2280-2289). Gnotobiotic beagle pups have also been infected with a human *H.pylori* isolate and kept under germ-free conditions

for 30 days. However, in this model no data are available on long term infections with *H.pylori* (Radin *et al.*, 1990). Other experimental animal models include athymic *nu/nu* or germ-free mice (Karita, M., *et al.* 1991. Am. J. Gastroenterol. 86:1596-1603).

The major drawbacks of these experimental infections, however, are the sophisticated and expensive housing systems required, and, more importantly, the peculiar immunological status of the gnotobiotic or immunodeficient hosts employed. More recently, *H.pylori*, freshly isolated from human gastroduodenal biopsies, have been adapted to persistently colonize the gastric mucosa of xenobiotic mice (Marchetti *et al.*, 1995). This model has proven particularly useful to assess the feasibility of either preventive (Manetti, R., *et al.* 1997. Infect. Immun. 65:4615-4619; Marchetti *et al.*, 1995; Marchetti, M., *et al.* 1998. Vaccine 16: 33-37; Radcliff, F.J., *et al.* 1997. Infect. Immun. 65:4668-4674) or therapeutic (Ghiara, P., *et al.* 1997. Infect. Immun. 65:4996-5002) vaccination, as well as for the *in vivo* screening of anti-*H.pylori* antimicrobials (Lee, A., *et al.* 1997. Gastroenterol. 112:1386-1397), and for studying the pathogenesis of infection (Sakagami, T., *et al.* 1996. Gut. 39:639-648). However, to evaluate gastric infection, mice have to be sacrificed; the pathological changes induced by the chronic infection and/or the effect of therapeutic or immunizing regimens cannot, therefore, be followed up in the same individual animal.

In United Kingdom patent application GB 9801000.2 (filed 16th January 1998) and associated International patent application PCT/IB99/00217 (filed 15th January 1999), there is described for the first time an animal model which can reproduce symptoms which have been clearly associated with the acute phases of infection with *H.pylori* in humans (Marshall, B.J., *et al.* 1985. Med. J. Australia. 142:436-439; Mitchell, J.D., *et al.* 1992. Am. J. Gastroenterol. 87:382-386; Morris, A., and G. Nicholson. 1987. Am. J. Gastroenterol. 82:192-199; Sobala, G.M., *et al.* 1991. Gut 32:1415-1418). The invention described in GB 9801000.2 and PCT/IB99/00217 is based on the discovery that *H.pylori* can persistently colonize the gastric mucosa of conventional xenobiotic dogs, and that this colonization causes acute symptoms, histopathological lesions and elicits specific immune responses. Thus, the animal model provided in GB 9801000.2 and PCT/IB99/00217 is ideal for studying the efficacy of treatments for *H.pylori* infection.

As *H.pylori* is a mucosa-related infection, where the bacteria do not invade the surrounding host cells, attempts to develop vaccines or treatments against the disease have concentrated on mucosal administration, specifically oral administration into the gastro-intestinal tract.

Thus, as it had previously been thought that local (mucosal) treatment at the site of the *H.pylori* infection was necessary, the thrust of research in this area has been to develop mucosa-associated anti-*H.pylori* antibodies by the mucosal administration of prophylactics/therapeutics (see, for example: Chen, M., *et al.* 1992. Lancet 339:1120-1121; Ferrero, R.L., *et al.* 1994. Infect. Immun. 62:4981-4989; Michetti, P., *et al.* 1994. Gastroenterology 107:1002-1011; Lee, A., *et al.* 1994. Infect. Immun. 62:3594-3597; Doidge, C., *et al.* 1994. Lancet 343:974-979; Marchetti *et al.*, 1995; Lee, C.K., *et al.* 1995. J. Infect. Dis. 172:161-172; Corthesy-Theulaz, I., *et al.* 1995. Gastroenterology 109:115-121; Cuenca, R., *et al.* 1996. Gastroenterology 110:1770-1775; Radcliff, F.J., *et al.* 1996. Vaccine 14:780-784; Stadtlander, C.T.K.H., *et al.* 1996. Dig. Dis. Sci. 41:1853-1862; Ferrero, R.L., *et al.* 1997. Gastroenterology 113:185-194; Weltzin, R., *et al.* 1997. Vaccine 15:370-376; Radcliff *et al.*, 1997; Ghiara *et al.*, 1997; Marchetti *et al.*, 1998).

However, it has been shown in the present invention that a systemic protective effect against challenge with infectious *H.pylori* can be unexpectedly achieved using a non-mucosally administrated *H.pylori* antigen-containing composition. Specifically, it has been shown that, for instance, intramuscular (i.m.) immunization with whole *H.pylori* cell lysate can protect dogs against challenge with infectious *H.pylori*, and that the i.m. route, as an example of a non-mucosal route, can, unexpectedly, be considered for vaccination against this bacterium. Such a method may also be useful therapeutically in treating an already established *H.pylori* infection.

All documents (including patents, patent applications, research articles and books) which are mentioned in this application are hereby incorporated in their entirety by reference.

Summary of the Invention

The present application is therefore based on the discovery that *H.pylori* antigen-containing compositions against *H.pylori* can be administered non-muscosally and still result in effective (systemic) protection from disease, despite the fact that the *H.pylori* bacteria are associated with the mucosa.

According to the present invention there is provided a method of protection against or treatment for infection by *H.pylori* comprising non-mucosal administration of an effective amount of one or more *H.pylori* antigens.

The term "comprising" means "including" as well as "consisting of".

Preferably, administration is parenteral, more preferably, intramuscular.

The one or more antigens administered may separately or in combination elicit a protective immune response in an animal, preferably, a mammal, more preferably a human.

At least one of the antigens may be a virulence factor of *H.pylori*. Examples of such virulence factors include VacA, CagA, NAP or urease. Other preferred antigens include HopX, HopY, 36kDa, 42kDa, and 17kDa (see WO 98/04702) or 50 kDa antigen (see EP-A-0793676). The virulence factors of *H. pylori* administered preferably include VacA, CagA, NAP and urease. More preferably, the virulence factors include VacA, CagA and NAP. Most preferably, the virulence factors include CagA and NAP.

The *H. pylori* antigens administered may consist of only virulence factors or of only specific combinations of virulence factors such as VacA, CagA, NAP and urease; VacA, CagA and NAP; or CagA and NAP.

The antigens administered may also include non-*H. pylori* antigens.

The one or more antigens is/are preferably purified antigen(s) or a whole cell immunogen. The term "purified" means that at least one step of purification has been carried out such that a purified antigen is more pure than the same antigen in its natural context. There may, however, be present some impurities associated with such purified antigens. The term "purified" includes the situation where an antigen is "isolated". In this case, the antigen is generally not associated with any other substance which may adversely affect its ability to protect against or treat infection by *H.pylori*. Thus, the term "isolated" implies the highest degree of purification.

The antigens are obtained by various usual methods, i.e. by purification/isolation from cell culture, recombinant technology or by chemical synthesis. The whole cell immunogen is preferably prepared by extraction from *H.pylori* cells. The extraction may be carried out by lysis or sonication of the *H.pylori* cells or any other suitable method. The whole cell immunogen may be or may comprise inactivated *H.pylori* cells.

An adjuvant is preferably co-administered with the one or more *H.pylori* antigens. Preferably the adjuvant is aluminium hydroxide or MF59™ (see WO 90/14837; EP-B-0399843; Ott *et al.* Chapter 10 of *Vaccine Design: The subunit and adjuvant approach*, eds. Powell & Newman, Plenum Press 1995)

The one or more antigens may also be in association with one or more pharmaceutically acceptable excipients.

The present invention also provides the use of an effective amount of one or more *H.pylori* antigens in the manufacture of a medicament for non-mucosal administration for protection against or treatment for infection by *H.pylori*.

The present invention further provides a pharmaceutical composition for non-mucosal administration comprising one or more *H.pylori* antigens and one or more pharmaceutically acceptable excipients. This is preferably an immunogenic composition.

The one or more antigens of the pharmaceutical composition may separately or in combination elicit a protective immune response in an animal, preferably a mammal, more preferably, a human.

Preferably, at least one of the antigens is a virulence factor of *H.pylori*. Examples of such virulence factors are given above, along with examples of other preferred antigens.

The pharmaceutical composition preferably includes at least the virulence factors of VacA, CagA, NAP and urease. More preferably, the pharmaceutical composition includes at least the virulence factors of VacA, CagA and NAP. Most preferably, the pharmaceutical composition includes at least the virulence factors of CagA and NAP.

The *H. pylori* antigens of the pharmaceutical composition may consist of only virulence factors or of only specific combinations of virulence factors such as VacA, CagA, NAP and urease, VacA, CagA and NAP, or CagA and NAP.

The antigens of the pharmaceutical composition may also include non-*H. pylori* antigens.

The one or more antigens is/are preferably purified antigen(s) or a whole cell immunogen as described above.

The pharmaceutical composition may also further comprise an adjuvant. Preferably, the adjuvant is aluminium hydroxide or MF59™.

The pharmaceutical composition of the invention can be an immunogenic composition such as, but not limited to, a vaccine.

The present invention therefore also provides an immunogenic composition for non-mucosal administration comprising one or more *H.pylori* antigens and one or more pharmaceutically acceptable excipients.

Immunogenic compositions and vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat disease after infection). Such vaccines comprise antigen or antigens, usually in combination with "pharmaceutically acceptable carriers," which include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminium salts (alum), such as aluminium hydroxide, aluminium phosphate, aluminium sulphate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components), such as for example (a) MF59™, containing 5% Squalene, 0.5% Tween™ 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE, although not required) formulated into submicron particles using a microfluidizer (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Freund's complete and incomplete adjuvants (CFA & IFA); (5) cytokines, such as interleukins (e.g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g. IFNγ), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; and (6)

other substances that act as immunostimulating agents to enhance the efficacy of the composition. Alum and MF59™ are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), *etc.*

The immunogenic compositions (*eg.* the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvanticity effect, as discussed above.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g. non-human primate, primate, etc.), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The immunogenic compositions are administered non-mucosally, more specifically parenterally *eg.* by injection, either subcutaneously or intramuscularly. Transdermal or transcutaneous administration may also be used. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

According to the present invention, there is further provided the use of the pharmaceutical composition as described above or the immunogenic composition as described above for protection against or treatment for infection by *H. pylori*.

Brief Description of the Drawings

Figure 1 shows endoscopic examination of the antral region of protected dogs (*Fig. 1A*) and control (infected) dogs (*Fig. 1B*) 42 days after challenge with *H.pylori*.

Figure 2 shows histological examination (hematoxylin-eosin (HE) staining) of biopsies taken from the antral region of protected dogs (*Fig. 2A*, magnification = 10x) and control (infected) dogs (*Fig. 2B*, magnification = 10x; *Fig. 2C*, magnification = 40x; *Fig. 2D*, magnification = 100x) 42 days after challenge with *H.pylori*.

Figure 3 shows immunohistochemical examination of biopsies taken from the antral region of protected dogs (*Fig. 3A*) and control (infected) dogs (*Fig. 3B*) 42 days after challenge with *H.pylori* (magnification = 40x).

Figure 4 shows the schedule of the experiment to determine whether Beagle dogs may be protected against a challenge with *H. pylori* using purified antigens given i.m.

Figure 5 shows serum antibody titers to recombinant VacA (*Fig. 5A*), to CagA (*Fig. 5B*) and to NAP (*Fig. 5C*) (averages) before the first immunization, and after the second, third and fourth immunizations i.m. with purified antigens or with whole cell lysate. The horizontal arrow individuates the lower limit for positive ELISA results.

Figure 6 shows the titers of serum IgG antibody subclasses IgG1 and IgG2 to CagA (*Fig. 6A*) and NAP (*Fig. 6B*) (average titers per groups immunized i.m. with purified antigens or with whole cell lysate) at the same time points as above (*Fig. 5*).

Figure 7 shows serum IgA antibody titers to p95 (recombinant VacA) in Beagle dogs immunized i.m. with purified antigens (VacA, CagA and NAP) or with whole cell lysate (single dogs – *Figs. 7A to 7F*) before immunization and after the third immunization. (Similar serum IgA antibody titers were observed against CagA.)

Figure 8 shows the endoscopic image of protected (*Fig. 8A*) and non-protected (infected) (*Fig. 8B*) dogs taken about 8 weeks post-challenge.

Figure 9 shows the histology (HE staining, magnification = 10x) of gastric mucosa from protected (*Fig. 9A*) and non-protected (infected) (*Fig. 9B*) dogs taken about 8 weeks post-challenge. Note in *Fig. 9A* the normal mucosa and submucosa. Conversely, in *Fig. 9B*, a large lymphoid follicle, disrupting the structure of the mucosa and the submucosa can be noted.

Figure 10 shows the immunohistochemistry using anti-VacA monoclonal antibody taken about 8 weeks post-challenge (magnification = 10x). Note the negative staining in protected dog (*Fig. 10A*) and the heavily positive staining in non-protected (infected) dog (*Fig. 10B*).

Detailed Description of the Invention

Whole cell H. pylori *vaccine*

As an embodiment of the invention, the feasibility of immunizing conventional dogs with whole cell *H.pylori* vaccine (i.e. *H.pylori* cell lysate) was investigated. It is shown that such a preparation can elicit an immunity that provides a protective effect against a later challenge with infectious *H.pylori*.

Immunogenicity. The protective effect of a whole cell *H.pylori* vaccine was investigated in dogs challenged with infectious *H.pylori*. The details of the experimental procedures followed are set out later. As compared to time 0 and to control dogs, i.m. immunization induced very high titers of serum IgG antibodies as shown in Table 1. I.m. immunization with *H.pylori* lysate also induced production of antigen-specific serum IgA antibodies.

Symptoms. Control dogs #2 and #3 had diarrhoea during the first week after the last challenge. Control dog #2 also had vomiting. There were no symptoms of *H.pylori* infection in the i.m. group.

Urease test on gastric (antral) bi psy and on gastric lavage. This assay, performed on antral biopsies and gastric lavage taken 10 days after the last challenge, was positive in the control group, and negative in the i.m.group as shown in Table 2 below, even 24 h later. These data were also confirmed at day 42 post-challenge.

Table 1

Immunogenicity of the H. pylori lysate given four times intramuscularly to Beagle dogs

Dog no.	Immunization route	Serum IgG titers to: H. pylori lysate Pre*	H. pylori lysate Post-4	CagA Pre	CagA Post-4	NAP Pre	NAP Post-4	Serum IgA titers to H. pylori lysate Pre	H. pylori lysate post-4
1	nil (control)	< 100§	< 100	< 100	< 100	< 100	< 100	< 20	< 20
2	nil (control)	< 100	< 100	< 100	< 100	< 100	< 100	< 20	< 20
3	nil (control)	< 100	< 100	< 100	< 100	< 100	< 100	< 20	< 20
4	intramuscular	< 100	≥ 25000	< 100	12800	< 100	≥ 12800	< 20	1280
5	intramuscular	< 100	≥ 25000	< 100	12800	< 100	≥ 12800	< 20	320
6	intramuscular	< 100	≥ 25000	< 100	6400	< 100	≥ 12800	< 20	640

*Pre: serum samples taken before the immunization; Post-4: serum sample taken after four immunizations, before the challenge.

§Titers are expressed as the last serum dilution giving an optical density equal or higher than 0.2. Titers < 100 (IgG) and <20 (IgA) are considered as negative.

Table 2

Group	*dog #*	*biopsy*	*gastric lavage*
Control	1	+	+
	2	+	+
	3	+	+
i.m.	1	-	-
	2	-	-
	3	-	-

Endoscopy, histology and immunohistochemistry results. Protected dogs showed normal mucosa at endoscopic examination at 42 days post-challenge (Fig. 1A), with a bright and smooth surface, without signs of hyperemia or edema. Conversely, in the control (infected) dogs, the gastric mucosa was heavily red and edematous, and had a rippled surface with appearance of plicae (Fig. 1B), suggestive of the nodular (follicular) gastritis observed previously and described in patent applications GB 9801000.2 and PCT/IB99/00217.

Histologically, the gastric mucosa of protected dogs conserved an intact structure, both at the surface and at the submucosa (Fig. 2A), whereas in infected dogs there was appearance of hyperemia (Fig. 2B, arrows), edema (Fig. 2B, asterisk), inflammatory cellular infiltrates (Figure 2B, arrowheads and Fig. 2C). *H.pylori* was also easily identified in the mucous layer (Fig. 2D).

All these data were confirmed by immunohistochemistry using an anti-VacA monoclonal antibody, which heavily stained epithelial cells of infected dogs (Fig. 3B, arrows), but not those of protected dogs (Fig. 3A).

C nclusions. Taken together these data show that i.m. immunization with *H.pylori* lysate can protect dogs against challenge with infectious *H.pylori*, and that the i.m. route can be considered for vaccination against this bacterium.

Purified H. pylori *antigen vaccine*

As a further embodiment of the invention, the feasibility of immunizing conventional dogs with purified *H.pylori* antigens was also investigated. It is shown that such a preparation can elicit an immunity that provides a protective effect against a later challenge with infectious *H.pylori*.

Immunogenicity. The protective effect of purified *H.pylori* antigens (specifically VacA, CagA and NAP) was investigated in dogs challenged with infectious *H.pylori*. The details of the experimental procedures followed are set out later. Immunization with these antigens induced very strong serum IgG antibody response specific for each antigen after only two doses. Titers increased after the third dose. Doses of 50 and 10 μg of antigens were as good as 250 μg to induce high titers of antigen-specific antibodies. Comparatively, lower antibody titers were detectable in dogs immunized with *H. pylori* lysate (see Fig. 5).

Immunization with these antigens also induced high titers of antigen-specific serum IgG1 and IgG2 antibodies, suggesting that this immunization induces both Th1- and Th2-type immune response, unlike what has already been observed in infected mice and dogs, in which a preponderant Th1-type immune response is evident (see Fig. 6).

Dogs immunized in this manner also had detectable titers of antigen-specific IgA antibodies in the serum (see Fig. 7).

Endoscopy, histology and immunohistochemistry results. In protected animals (see Table 3 – which gives the conclusive results on protection, based on all parameters taken together, including endoscopy (gastroscopy), histology and immunohistochemistry), gastric mucosa was normal at endoscopic investigation, at histology, and at immunohistochemistry (see Figs. 8A, 9A and 10A, respectively). Non-protected (infected) animals showed hyperemic, heavily flogistic aspect of the gastric mucosa at endoscopy (Fig. 8B), with a diffuse infiltration with mononuclear cells aggregated in lymphoid follicle structures (Fig. 9B) disrupting the normal glandular structure. Fig. 10B shows a strong positivity at immunohistochemistry using an anti-VacA monoclonal antibody.

C nclusions. Taken together these data show that i.m. immunization with *H. pylori* antigens can protect dogs against challenge with infectious *H.pylori*.

Table 3

Intramuscular immunization with purified antigens protects dogs against challenge with *Helicobacter pylori*

Group	Dog #	Protection
Control	1	-
	2	-
	3	-
	4	-
VacA/CagA/NAP	5	+
250 µg each	6	+
	7	+
	8	-
VacA/CagA/NAP	9	+
50 µg each	10	+
	11	+
	12	+
VacA/CagA/NAP	13	+
10 µg each	14	+
	15	+
	16	+
whole-cell lysate	17	-
25 mg	18	+
	19	+
	20	+
whol -c ll lysat	21	+
5 mg	22	+
	23	-
	24	+

Experimental

***H.pylori* strains.** SPM326s, a streptomycin-resistant derivative of the mouse-adapted *H.pylori* Type I (CagA+/VacA+) strain SPM326 (Marchetti *et al.*, 1995), was grown as previously described (Marchetti *et al.*, 1995) and used to challenge the dogs. The CCUG strain of *H.pylori* is well known in the art.

Animals. Six 4-6 months-old xenobiotic beagle dogs, all female (Morini s.a.s., S. Polo D'Enza, Italy), were selected on the basis of the absence of detectable serum IgG against *Helicobacter spp.* in Western blot (WB) analysis using total bacterial lysate as antigen (see below). The six dogs selected were housed in standard conditions and maintained on a diet of dry food (MIL, Morini s.a.s.) and tap water *ad libitum*. Upon arrival in our animal facilities, an additional WB analysis on sera confirmed their *H.pylori* status. The dogs were housed in individual cages and allowed to adapt for a month to the new environment. During the month of adaptation, two tests were carried out on fecal samples to assess the presence of intestinal parasites or common enteric pathogenic bacteria.

Preparation of *H.pylori* lysate. Two pellets of *H.pylori* CCUG strain from two 5 liter fermenters (Olivieri, R., *et al.* 1993. J. Clin. Microbiol. 31:160-162) were obtained. After each pellet was resuspended in 50 ml of sonication buffer (50 mM $Na_2HPO_4.2H_20$, 300 mM NaCl, pH 7.8), the two resuspended pellets were mixed. The OD_{530nm} of the combined resuspension was measured to determine bacterial concentration (= 3.2 x 10^{10} CFU/ml). The resuspension was diluted with sonication buffer to bring the concentration to 2 x 10^{10} CFU/ml. Before sonication, bacteria had the classical spiral form when viewed under the microscope. Sonication of the resuspended bacteria was then carried out on ice: 2 cycles of 4 minutes, and 2 cycles of 5 minutes, waiting one minute between each cycle. After sonication, all the bacteria appeared broken when viewed under the microscope. Protein concentration was then determined using the Bradford method (= 57.5 mg/ml protein). Aliquots of the cell lysate were then prepared and frozen at -80°C until use.

Immunization. Three dogs were immunized on day 0 intramuscularly (i.m.) with the prepared *H.pylori* lysate (the equivalent of 10^{10} CFU *H.pylori* (= 28 mg/dose)) adsorbed onto 1 mg aluminium hydroxide (Chiron Behring GmbH & Co., Marburg, Germany; Lot No. 277345) in 1 ml volume. Immunizations were repeated on days 7, 14, and 22. Serum samples were taken on day 0, 21 (post-3) and 43 (post-4). The other three dogs, as a control group, were treated identically but saline was used in place of the *H.pylori* lysate.

Challenge with infectious *H.pylori*. The dogs were then challenged on days 49, 51 and 53 with the mouse-adapted *H.pylori* SPM326s strain as follows: 24 h before each challenge the dogs were fasted. 2 h before bacteria inoculation, dogs received 10 mg/kg of cimetidine i.m. (Tagamet® 200; Smith Kline & French, USA). At the moment of challenge, the dogs were anesthetized with a mixture of 40 μg/kg of medetomidine chloridrate (Domitor®; Centralvet-Vetem s.p.a., Milano, Italy) and 5 mg/kg of ketamine (Ketavet®, Gellini, Latina, Italy) intravenously (i.v.); then a gastric lavage was performed with 100 ml of 0.2M $NaHCO_3$ sterile solution followed by oral challenge with 3 ml of a freshly prepared suspension of 10^9 CFUs in sterile saline of the *H.pylori* strain SPM326s, grown under microaerobic conditions (see below), prepared immediately before the inoculation procedure. At the end of the bacterial inoculation, 200 μg/kg of the anesthetic antagonist atipamezole (Antisedan®; Centralvet-Vetem s.p.a., Milano, Italy) was administered and then dogs were again treated with cimetidine and fed after 2 h.

Post-challenge follow-up. Ten and 42 days after the last challenge gastric endoscopies were performed using a 4.9-mm-diameter Pentax pediatric bronchoscope (Pentax Technologies, Zaventem, Belgium). At the same time, gastric biopsies were taken during the endoscopies using flexible pinch-biopsy forceps at the antrum, corpus fundus, and cardias for urease testing and for microbiological, histopathological and immunohistochemical analyses. Before each endoscopy the whole instrument and the flexible forceps were soaked in 4% glutaraldehyde for 45 minutes and then rinsed in sterile saline. To avoid cross-contamination among biopsies taken at different sites, the forceps were washed with tap water and lightly flame-sterilized before the collection of each bioptic sample. The above experimental protocol was approved by the Scientific and Ethical Commitee of the University of Pisa and received official authorization (DM No. 21/97-C) from the Italian Ministry of Health (Department of Veterinary Health, Food and Nutrition).

Rapid urease test. Antral biopsies and liquid from gastric lavage were incubated for up to 24 h in 1 ml of a 10% urea solution in distilled water added with two drops of a 1% phenol red solution (Sigma Chemical Co., St. Louis, MO, USA) in sodium phosphate buffer, pH 6.5. A positive test is indicated by change of color (from orange to dark pink) in the medium; the time necessary for the color change is recorded. At time 0, endoscopy was

carried out on the six dogs and antral biopsies were taken for the urease test. In all six dogs, the urease test was negative at time 0.

Histopathology and Transmission Electron Microscopy. Samples for histological, immunohistochemical and ultrastructural examination were taken from the biopsies at sites adjacent to those utilized for microbiological analysis. The samples were fixed in 10% buffered formalin and embedded in paraffin. 3 μm sections were stained with hematoxylin-eosin (H & E) and Alcian and Periodic acid Schiff's (PAS) staining using standard procedures for histopathological examination. Similar sections were also employed for immunohistochemical analyses using the Avidine-Biotine-Complex (ABC)-peroxidase technique with a monoclonal antibody (Mab) specific for *H.pylori* (Biogenesis Ltd, Poole, England, UK) or an anti-VacA mouse monoclonal antibody (C1G9) obtained by immunizing Balb/c mice with purified native *H.pylori* VacA (Burroni and Telford, unpublished observations). Biotinylated horse anti-mouse antibody was used as secondary antibody. The reaction was developed with 3-1-diaminobenzidine-chlorhydrate (DAB) (Sigma) for identification and location of bacterial antigen. For electron microscopic examination, other samples were fixed in Karnowsky, post-fixed in OsO_4, and embedded in Epon-Araldite (Polysciences Inc., Warrington, PA, USA). Semi-thin sections were stained with toluidine blue for evaluation of cell damage, whereas ultra-thin sections were stained with uranyl acetate and lead citrate, and then examined with a Philips EM 301 transmission electron microscope (TEM) operating at 80 KV.

Detection of anti-*H.pylori* antibodies. SDS-PAGE of *H.pylori* (strain SPM326s) and WB analysis of sera were performed according to previously published procedures (Marchetti *et al.*, 1995). Briefly, dog sera were diluted 1:200 and incubated for 2 h at room temperature. Then, horseradish peroxidase (HRP)-conjugated rabbit anti-dog IgG antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) was added at 1:2,000 dilution for 2 h, and the reaction was developed using 4-α-chloronaphtol as substrate. Detection of antibody against *H.pylori* by ELISA was carried out on 96-well plates coated overnight at 4°C with the prepared CCUG strain lysate used for immunization (5 μg/well) or with purified native CagA or NAP (0.2 μg/well). Coated wells were blocked with PBS containing 5% non-fat milk. Twofold serial dilutions of the sera were incubated at 37°C for 2 h and then washed with PBS. Antigen specific IgG titers were determined using a 1:4,000 dilution of HRP-conjugated goat anti-dog IgG antibody (Bethyl Laboratories, Inc.,

Montgomery, TX, USA) for 2 h at 37°C. Antigen bound antibodies were revealed by adding o-phenylenediamine dihydrochloride (Sigma) as a substrate. Antibody titers were determined as previously described (Ghiara *et al.*, 1997).

Intramuscular (i.m.) immunization of dogs with purified *H. pylori* antigens. VacA and CagA were expressed and purified as described in Ghiara, *et al.* 1997. NAP was expressed and purified as described in earlier patent applications GB 9807721.7 and PCT/IB99/00695. As shown in Table 4, groups of 4 dogs were immunized i.m. with:

(i) a mixture of recombinant VacA, CagA, and NAP (250, 50 or 10 μg of each antigen) adsorbed onto aluminium hydroxide (1 mg dose);

(ii) CCUG lysate (prepared as discussed above), at 25 or 5 mg per dose adsorbed onto aluminium hydroxide (1 mg dose);

(iii) aluminium hydroxide alone (1 mg dose).

Dogs were immunized 4 times at weekly intervals. Challenge with *H. pylori* was carried out 4 weeks later as already described. Samples (blood, biopsies, *etc.* – as previously described) were taken before immunization, after the second, third and fourth immunizations, and then 2-3 weeks and 8 weeks after the last challenge.

Table 4

IMMUNIZATION PROTOCOL

All antigens given intramuscularly with alum as adjuvant

GROUP	DOGS	ROUTE	IMMUNOGEN	DOSE	ADJUVANT (mg)	VOLUME
1	4	control (IM)	nil (saline)	-	AlOH (1 mg)	1 ml
2	4	IM	VacA + CagA + NAP	250 μg each (tot. 750 μg)	AlOH (1 mg)	1 ml
3	4	IM	VacA + CagA + NAP	50 μg each (tot. 150 μg)	AlOH (1 mg)	1 ml
4	4	IM	VacA + CagA + NAP	10 μg each (tot. 30 μg)	AlOH (1 mg)	1 ml
5	4	IM	CCUG lysate	25 mg	AlOH (1 mg)	1 ml
6	4	IM	CCUG lysate	5 mg	AlOH (1 mg)	1 ml

Claims

1. A method of protection against or treatment for infection by *H.pylori* comprising non-mucosal administration of an effective amount of one or more *H.pylori* antigens.

2. The method of claim 1, wherein administration is parenteral.

3. The method of claim 2, wherein administration is intramuscular, transdermal or transcutaneous.

4. The method of any one of claims 1 to 3, wherein the one or more antigens separately or in combination elicit a protective immune response in an animal.

5. The method of claim 4, wherein the animal is a mammal, preferably a human.

6. The method of any one of claims 1 to 5, wherein at least one of the antigens is a virulence factor of *H.pylori*.

7. The method of claim 6, wherein the virulence factor of *H.pylori* is VacA, CagA, NAP or urease.

8. The method of any one of claims 1 to 7, wherein the one or more antigens is/are purified antigen(s) or a whole cell immunogen.

9. The method of claim 8, wherein the whole cell immunogen is prepared by extraction from *H.pylori* cells, preferably by lysis or sonication of the *H.pylori* cells.

10. The method of claim 8 or claim 9, wherein the whole cell immunogen is or comprises inactivated *H.pylori* cells.

11. The method of any one of claims 1 to 10, wherein an adjuvant is co-administered with the one or more *H.pylori* antigens.

12. The method of claim 11, wherein the adjuvant is aluminium hydroxide or MF59™.

13. The method of any one of claims 1 to 12, wherein the one or more antigens is/are in association with one or more pharmaceutically acceptable excipients.

14. The use of an effective amount of one or more *H.pylori* antigens in the manufacture of a medicament for non-mucosal administration for protection against or treatment for infection by *H.pylori*.

15. A pharmaceutical composition for non-mucosal administration comprising one or more *H.pylori* antigens and one or more pharmaceutically acceptable excipients.

16. The pharmaceutical composition of claim 15, wherein the one or more antigens separately or in combination elicit a protective immune response in an animal.

17. The pharmaceutical composition of claim 16, wherein the animal is a mammal, preferably a human.

18. The pharmaceutical composition of any one of claims 15 to 17, wherein at least one of the antigens is a virulence factor of *H.pylori*.

19. The pharmaceutical composition of claim 18, wherein the virulence factor of *H.pylori* is VacA, CagA, NAP or urease.

20. The pharmaceutical composition of claim 19, which includes at least the virulence factors of VacA, CagA and NAP.

21. The pharmaceutical composition of claim 19 or claim 20, which includes at least the virulence factors of CagA and NAP.

22. The pharmaceutical composition of any one of claims 15 to 21, wherein the one or more antigens is/are purified antigen(s) or a whole cell immunogen.

23. The pharmaceutical composition of claim 22, wherein the whole cell immunogen is prepared by extraction from *H.pylori* cells, preferably by lysis or sonication of the *H.pylori* cells.

24. The pharmaceutical composition of claim 22 or claim 23, wherein the whole cell immunogen is or comprises inactivated *H.pylori* cells.

25. The pharmaceutical composition of any one of claims 15 to 24 further comprising an adjuvant.

26. The pharmaceutical composition of claim 25, wherein the adjuvant is aluminium hydroxide.

27. An immunogenic composition for non-mucosal administration comprising one or more *H. pylori* antigens and one or more pharmaceutically acceptable excipients.

28. The use of the pharmaceutical composition of any one of claims 15 to 26 or the immunogenic composition of claim 27 for protection against or treatment for infection by *H.pylori*.

FIG. 1A

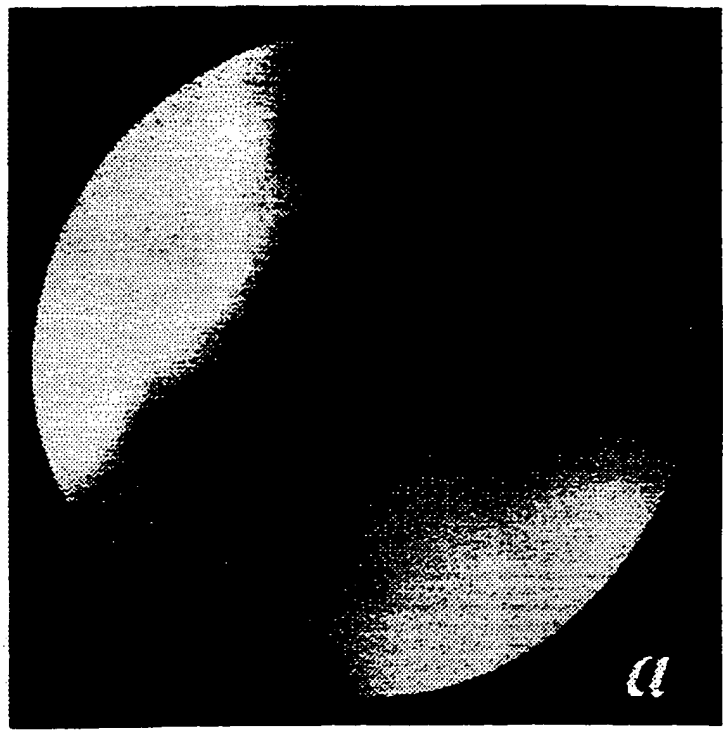


FIG. 1B

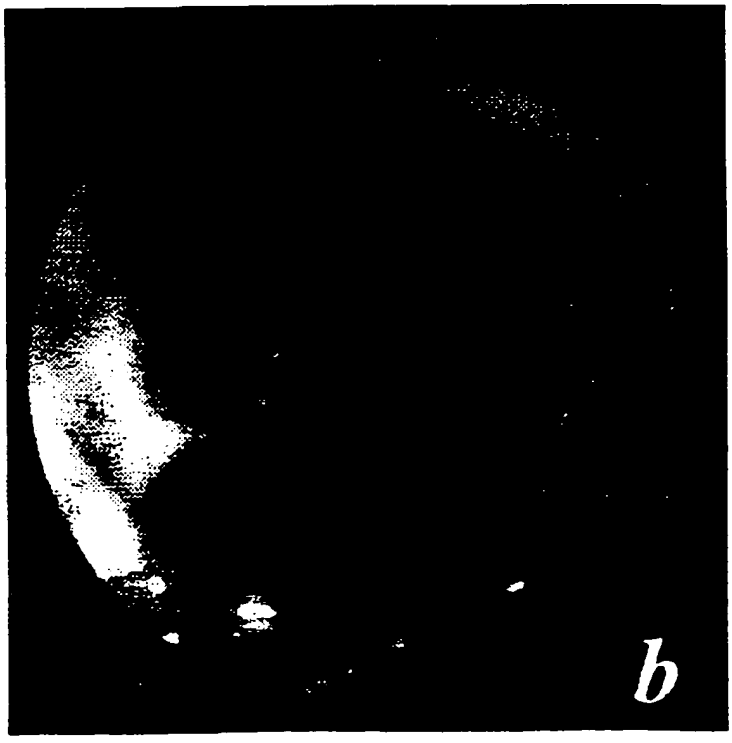


FIG. 2A

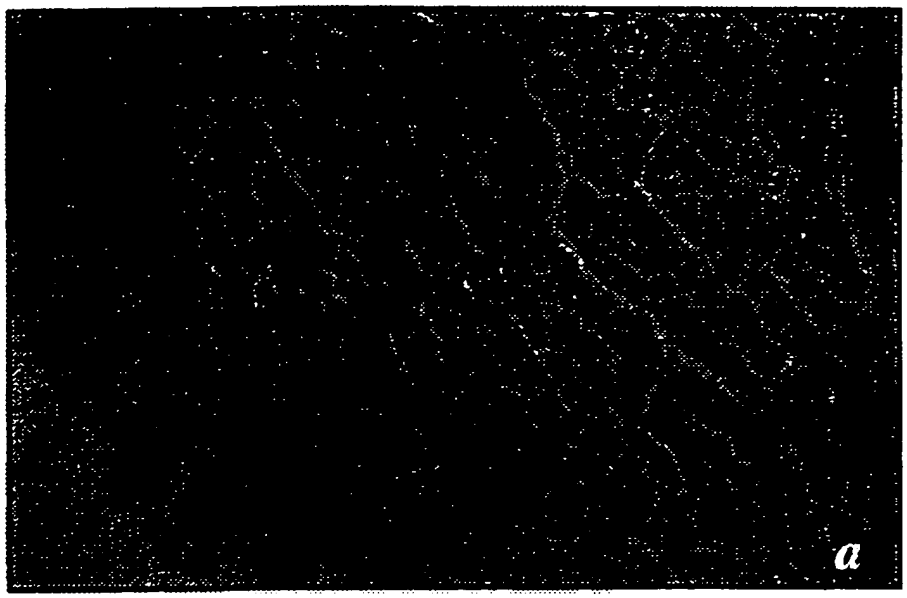


FIG. 2B

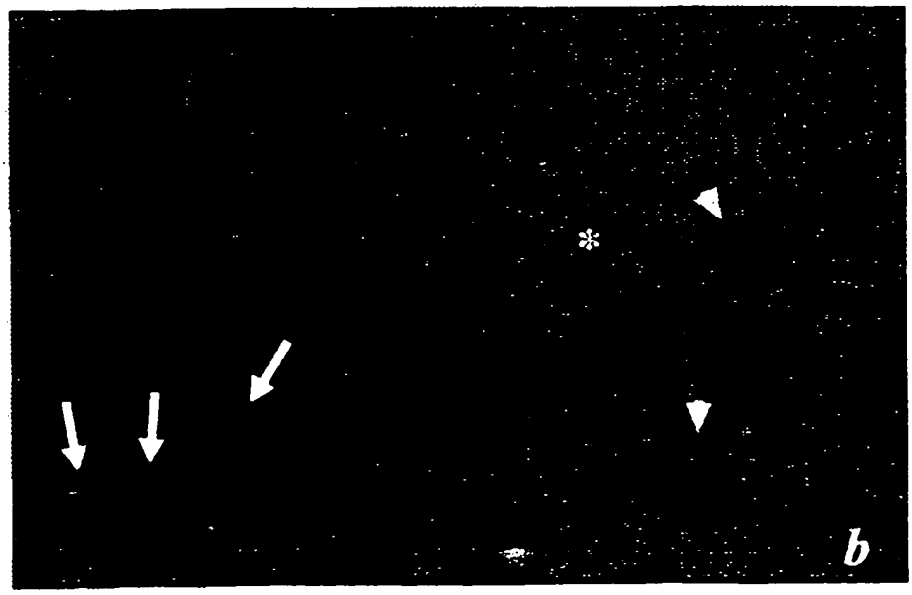


FIG. 2C

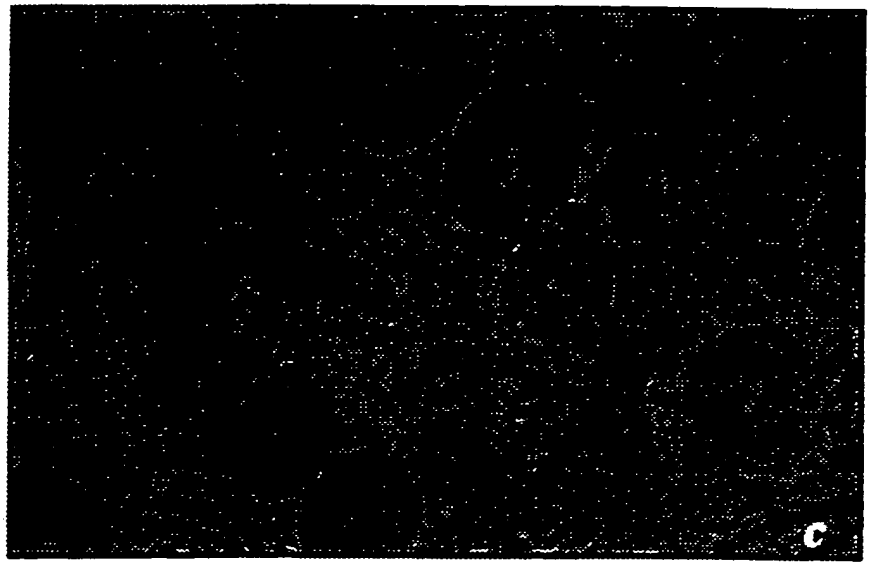


FIG. 2D

FIG. 3A

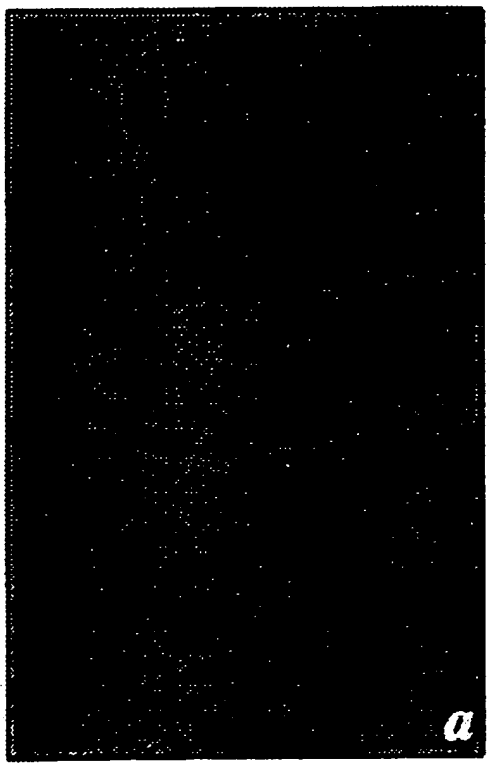


FIG. 3B

FIG. 4

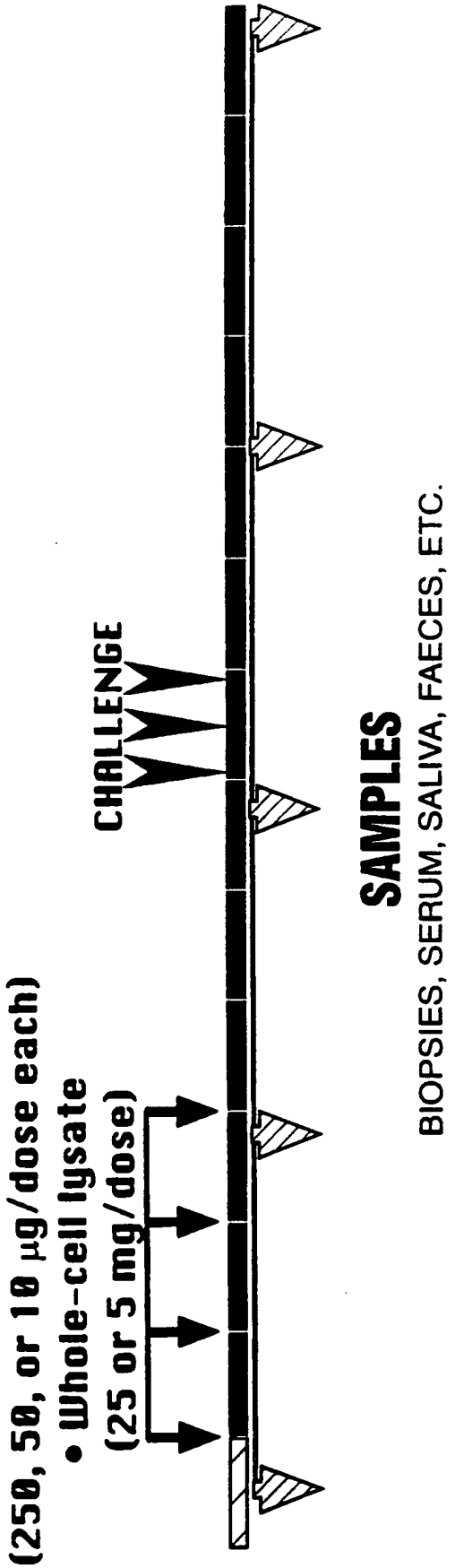

IMMUNIZATIONS
VacA, CagA & NAP
(250, 50, or 10 μg/dose each)
• Whole-cell lysate
(25 or 5 mg/dose)
CHALLENGE
SAMPLES
BIOPSIES, SERUM, SALIVA, FAECES, ETC.

FIG. 5A

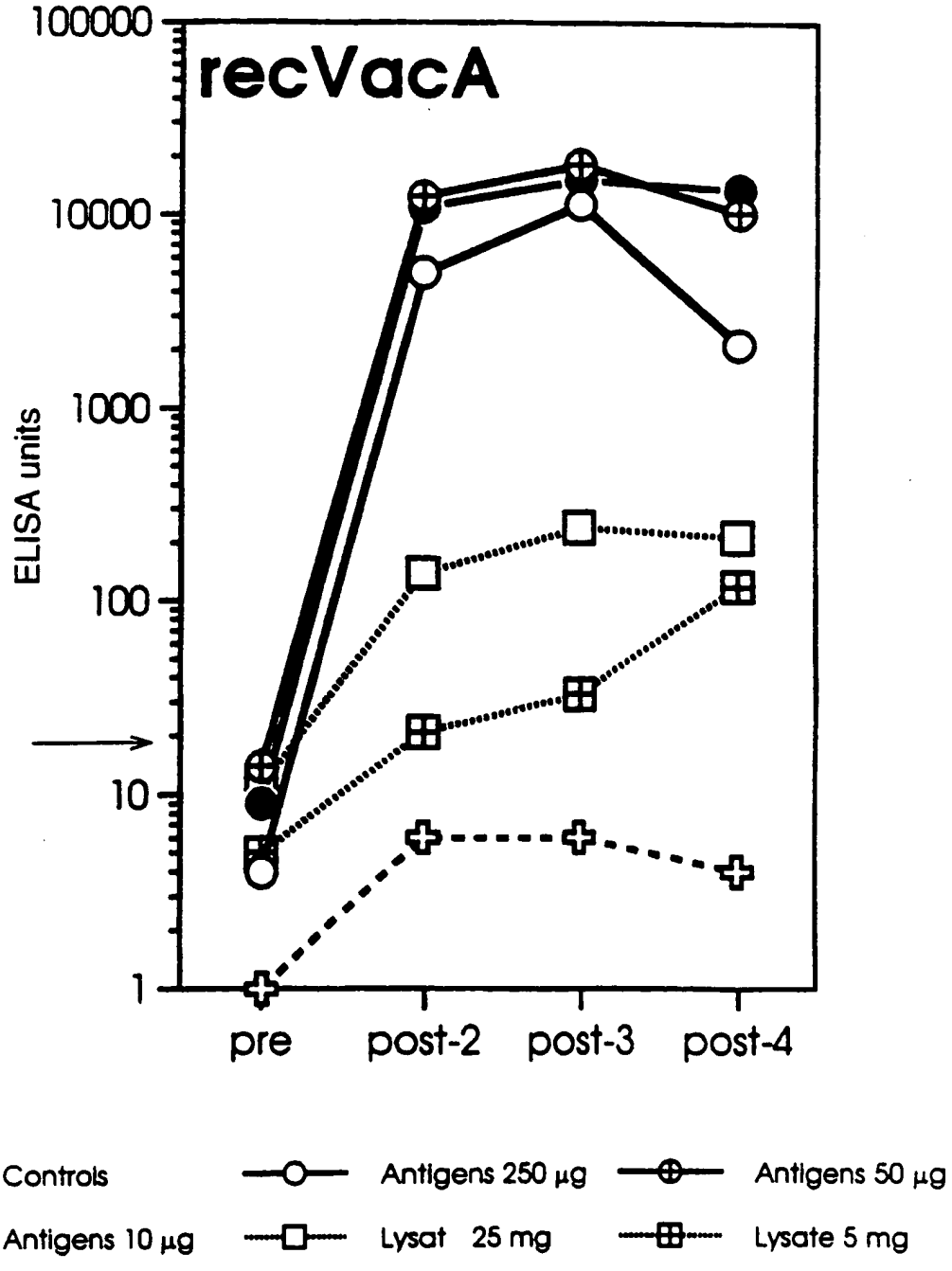

recVacA
100000
10000
1000
100
10
1
ELISA units
pre
post-2
post-3
post-4
Controls
Antigens 250 µg
Antigens 50 µg
Antigens 10 µg
Lysat 25 mg
Lysate 5 mg

FIG. 5B

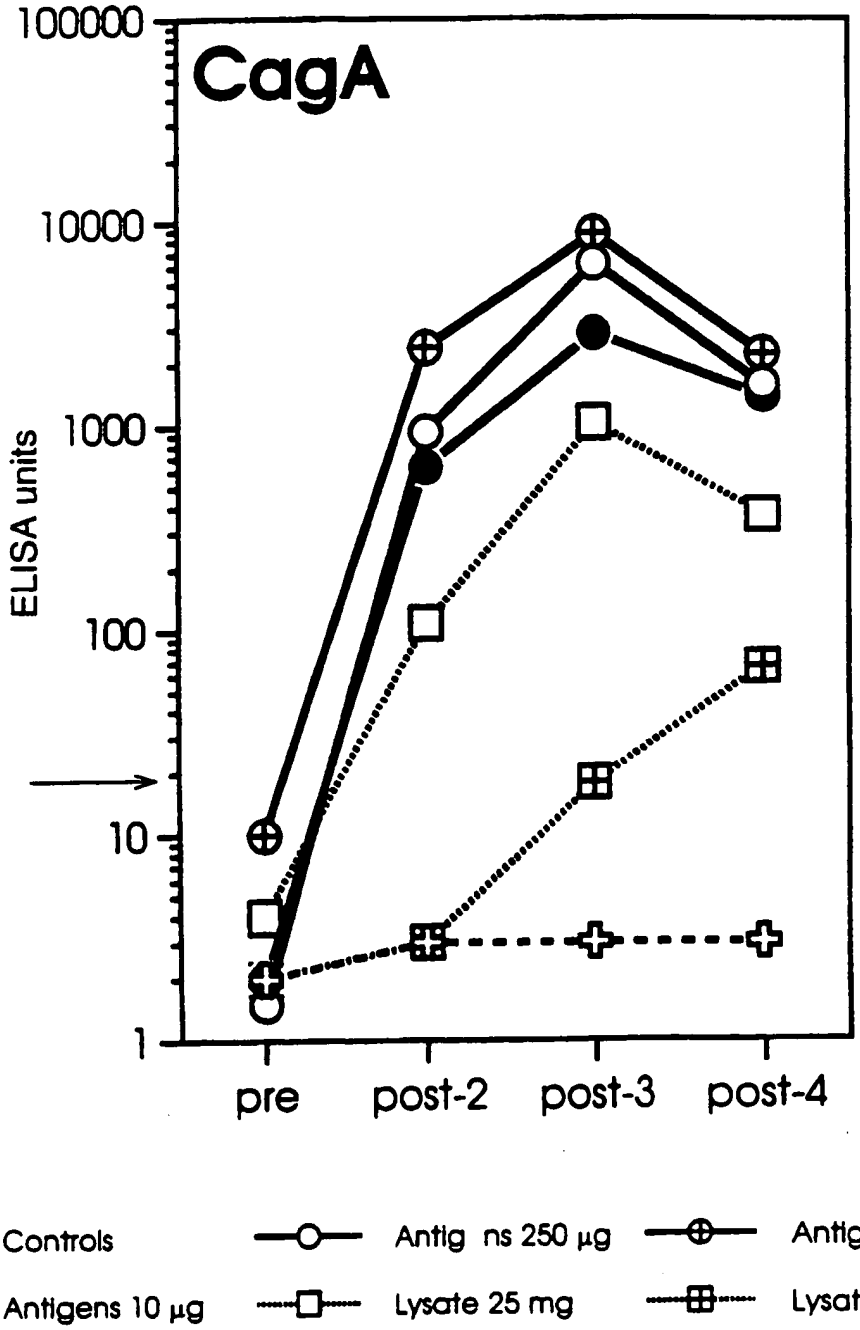
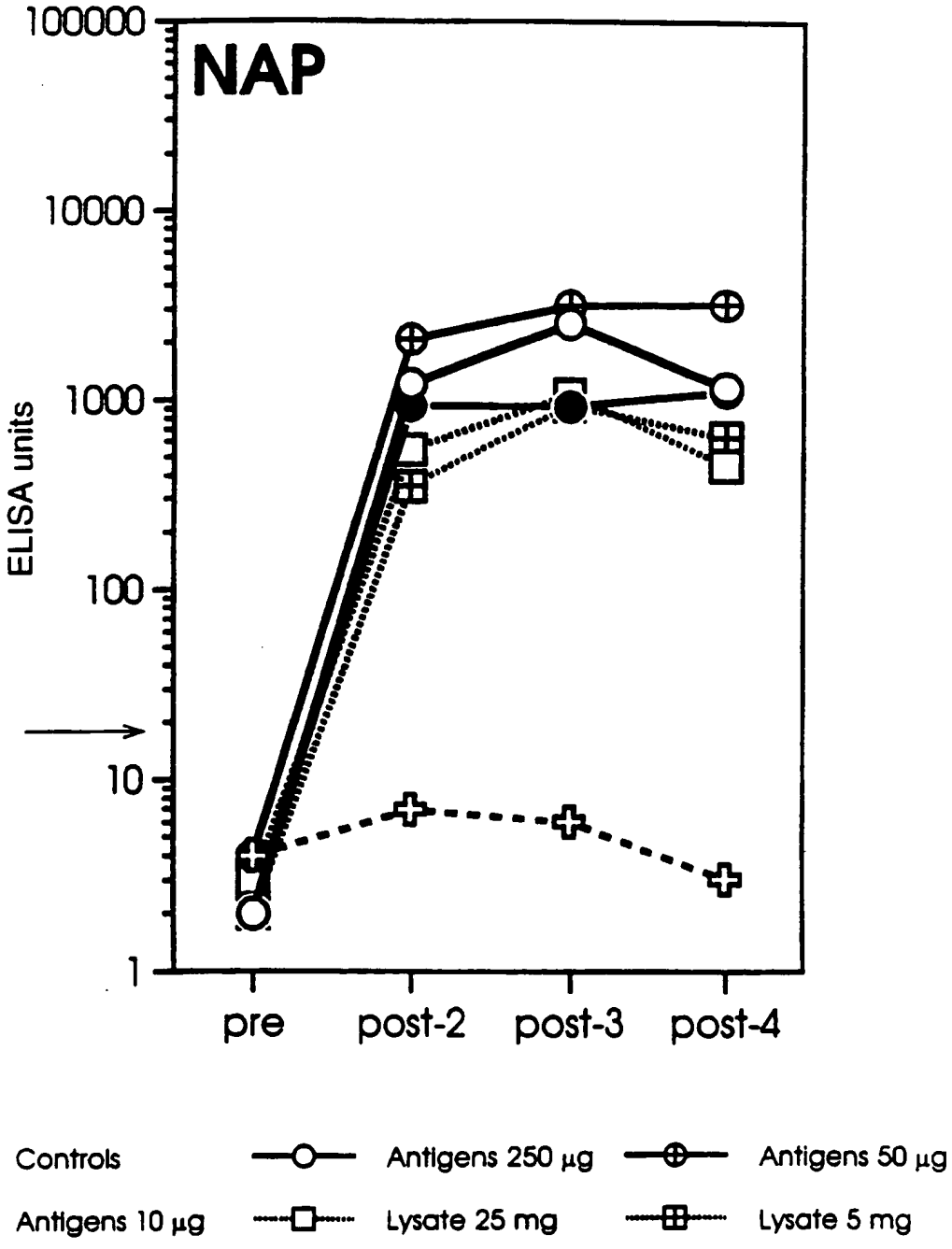

CagA
100000
10000
1000
100
10
1
ELISA units
pre
post-2
post-3
post-4
Controls
Antigens 10 µg
Antigens 250 µg
Lysate 25 mg
Antigens 50 µg
Lysate 5 mg

FIG. 5C


NAP
100000
10000
1000
100
10
1
ELISA units
pre
post-2
post-3
post-4
Controls
Antigens 250 µg
Antigens 50 µg
Antigens 10 µg
Lysate 25 mg
Lysate 5 mg

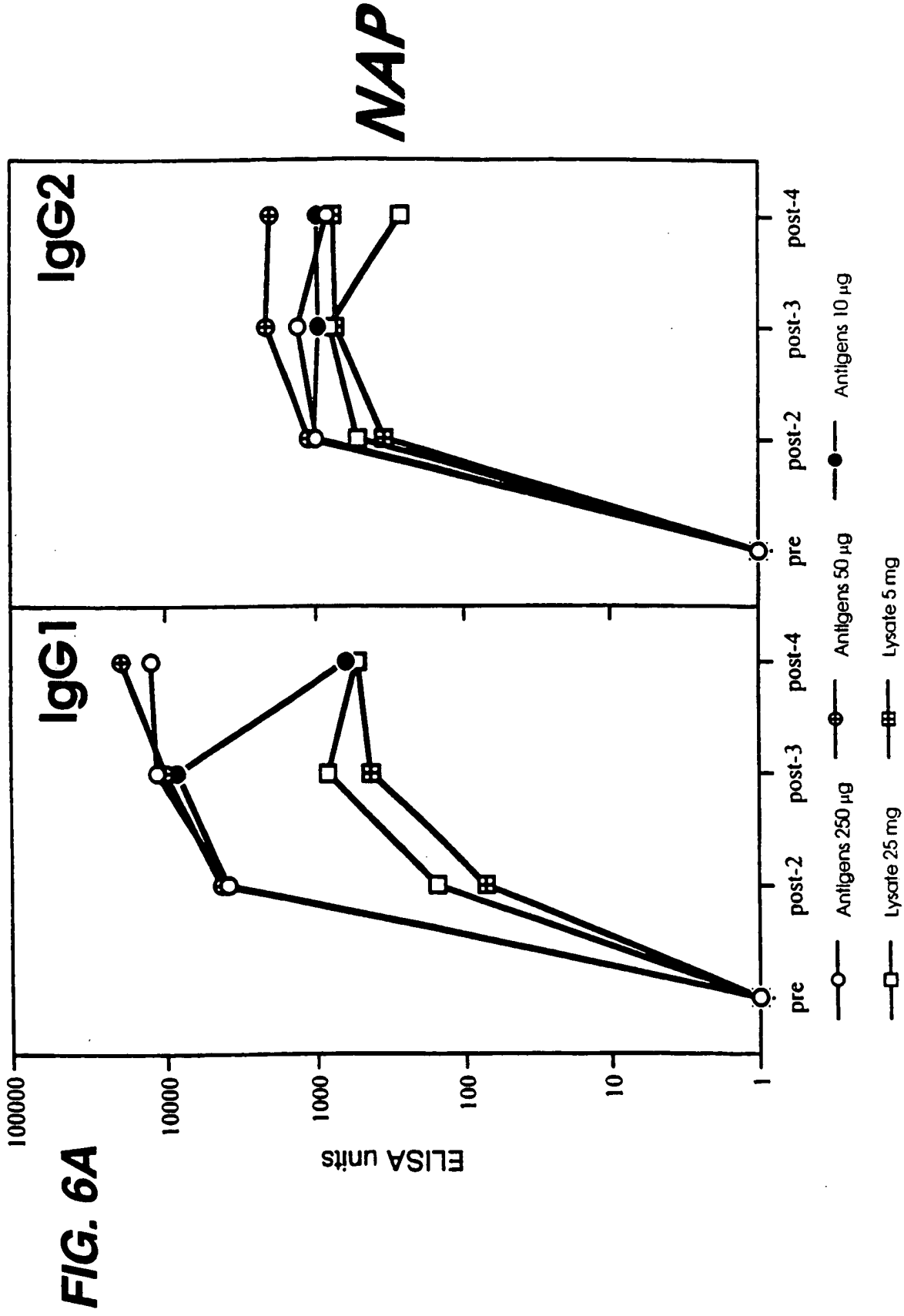

NAP
IgG2
IgG1
ELISA units
100000
10000
1000
100
10
1
pre
post-2
post-3
post-4
Antigens 250 µg
Antigens 50 µg
Antigens 10 µg
Lysate 25 mg
Lysate 5 mg

FIG. 6A

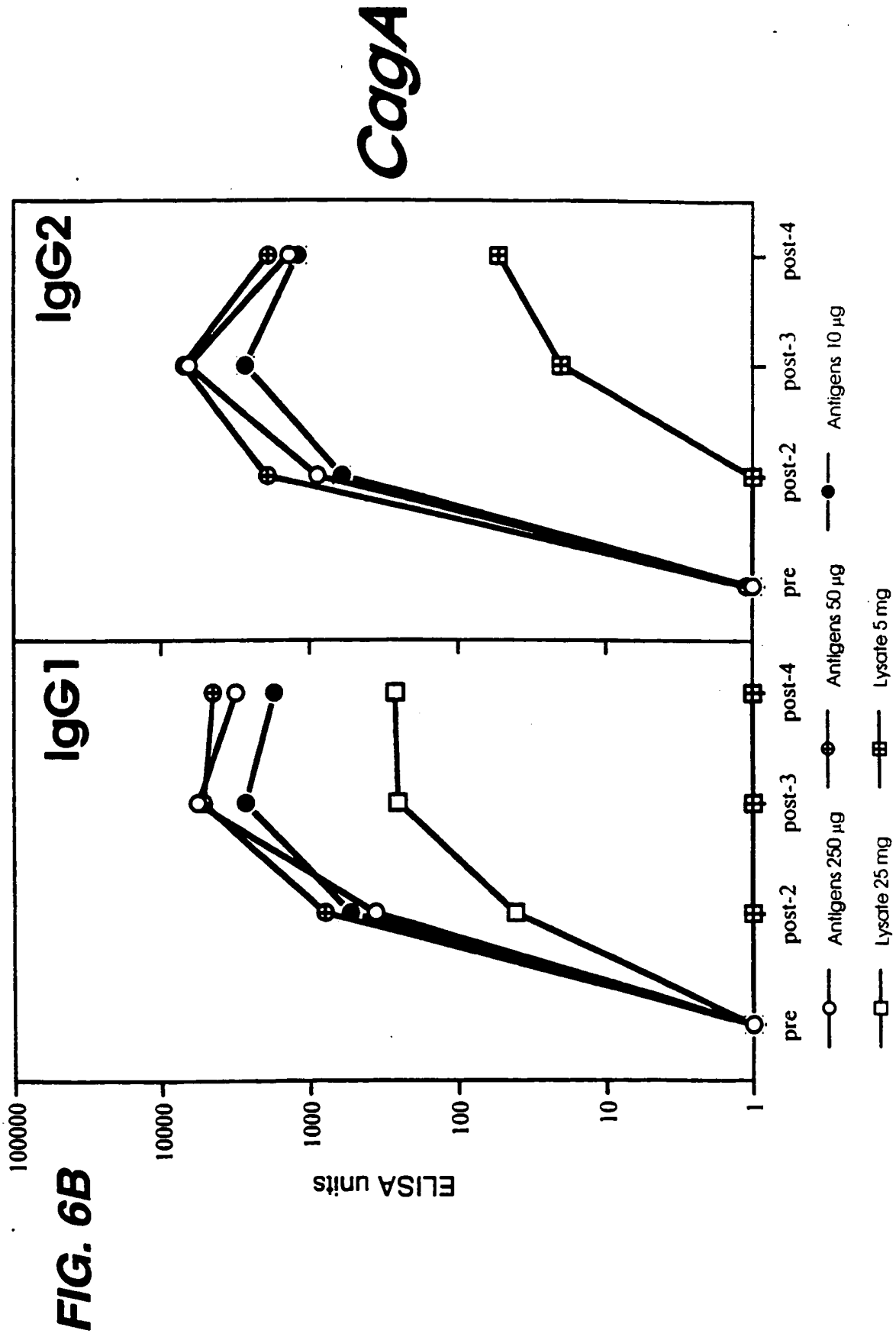

CagA
IgG2
IgG1
ELISA units
100000
10000
1000
100
10
1
pre
post-2
post-3
post-4
pre
post-2
post-3
post-4
Antigens 250 µg
Antigens 50 µg
Antigens 10 µg
Lysate 25 mg
Lysate 5 mg

FIG. 6B

FIG. 7A

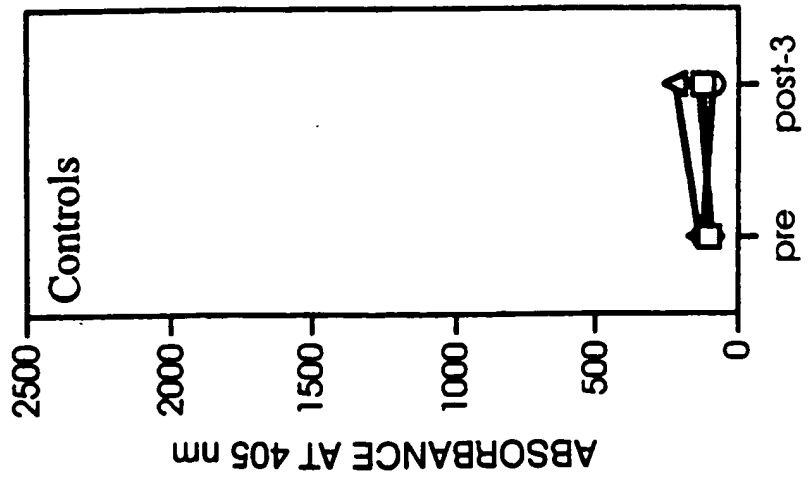


FIG. 7B

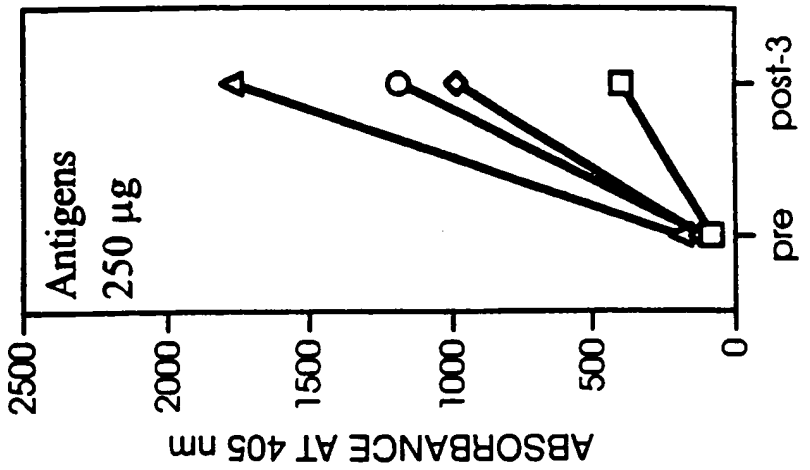


FIG. 7C

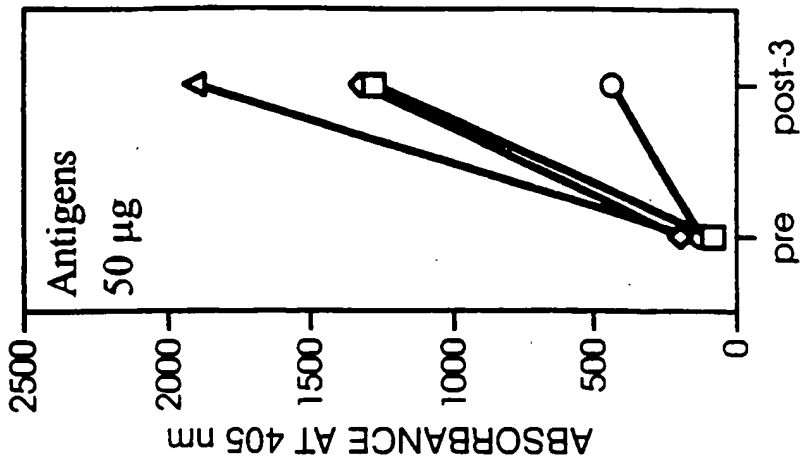


FIG. 7D

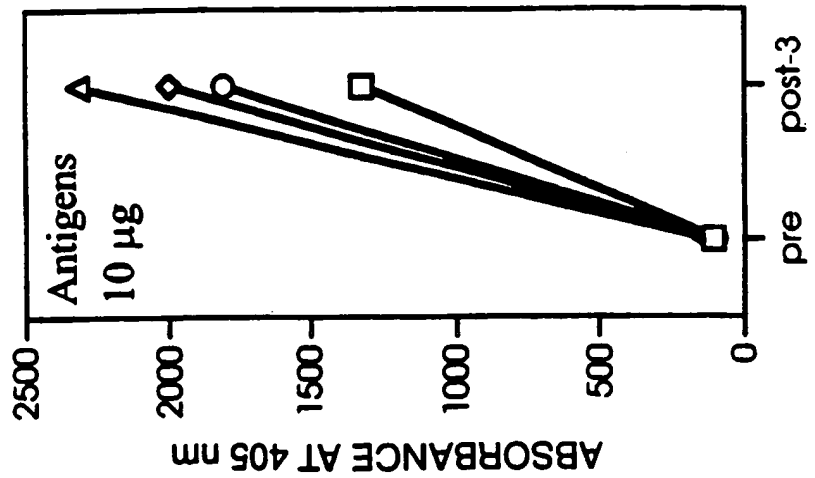


FIG. 7E

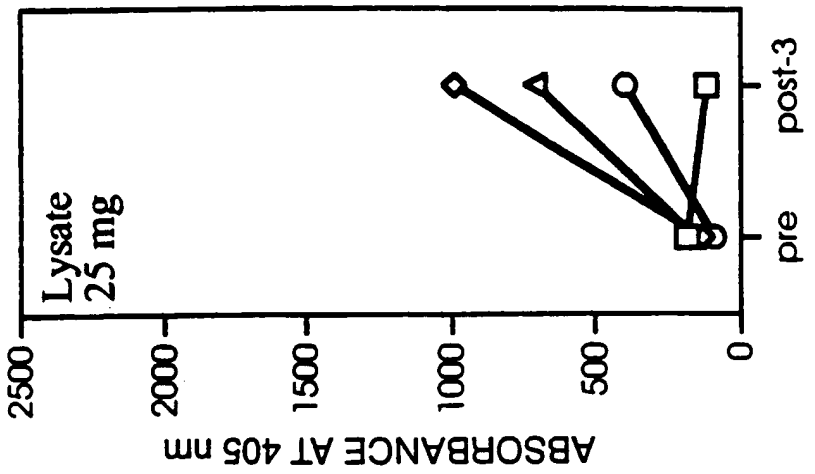


FIG. 7F

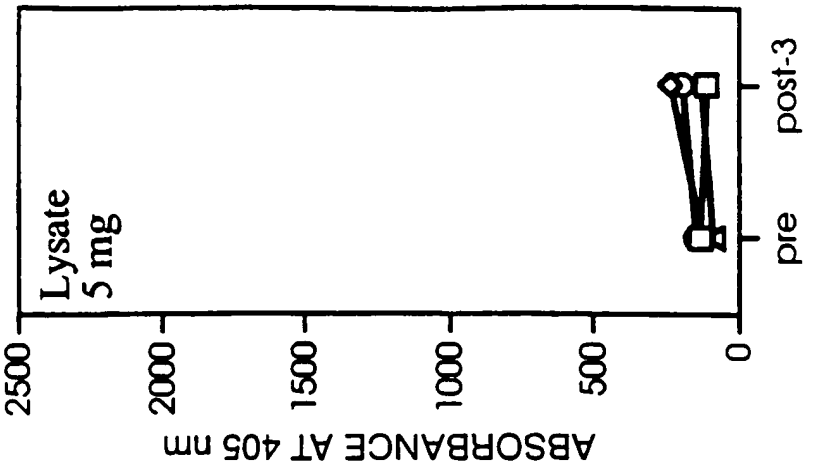


FIG. 8A

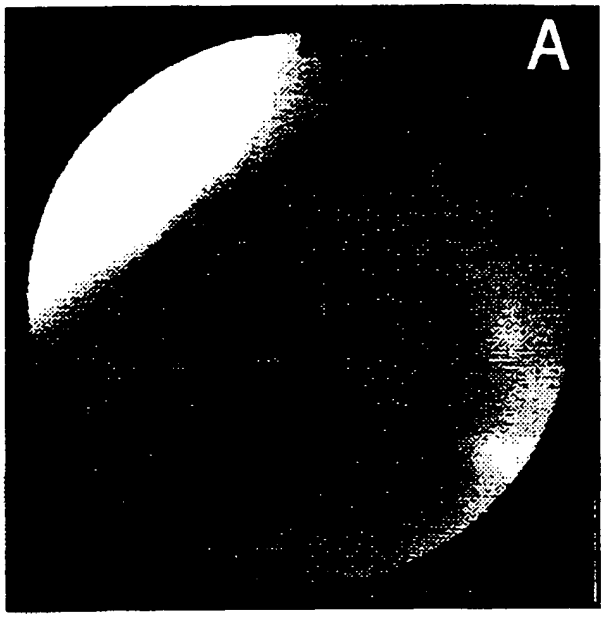


FIG. 8B

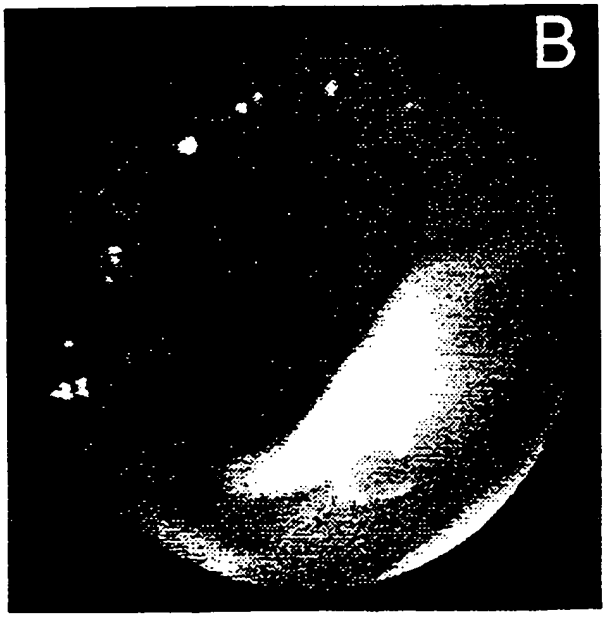


FIG. 9B

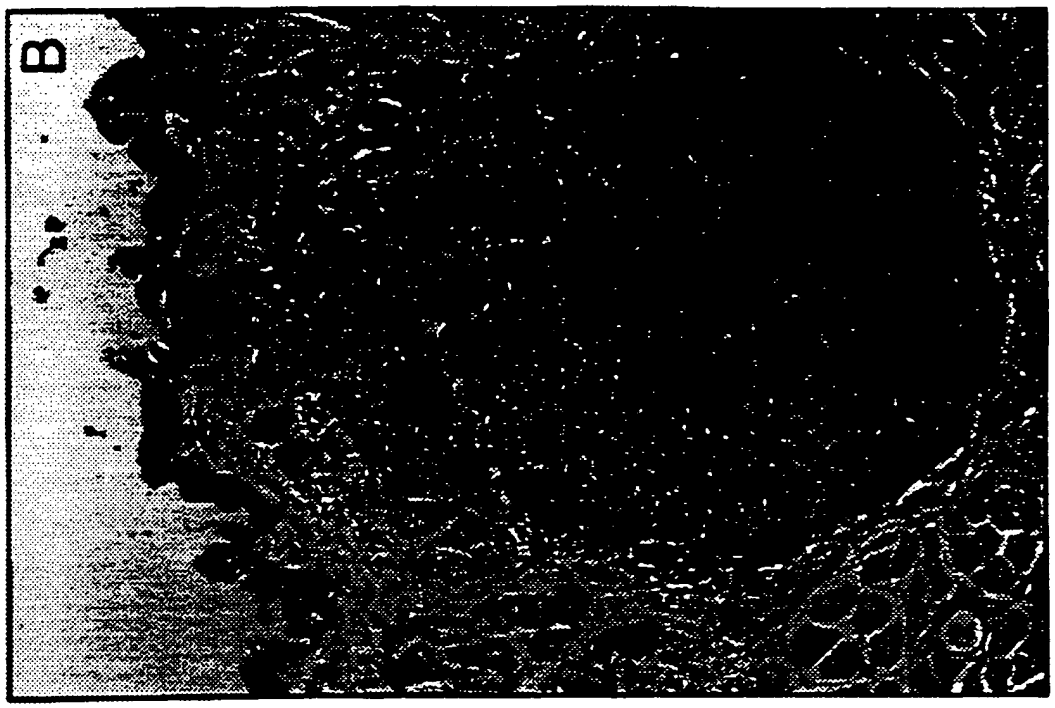


FIG. 9A

FIG. 10A

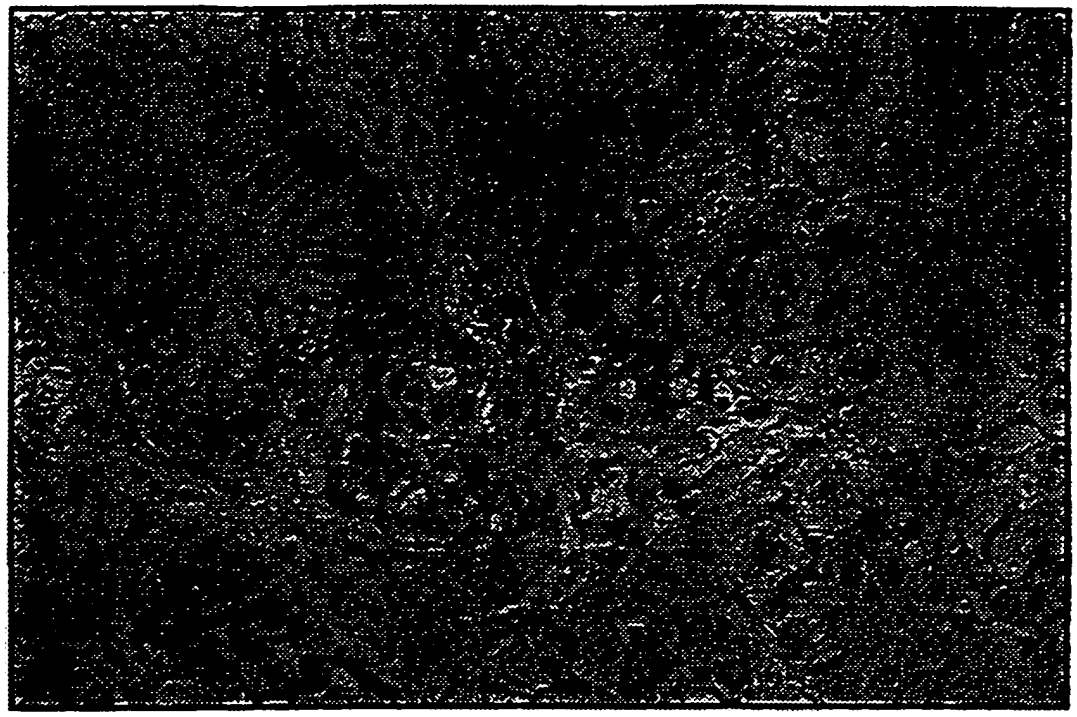


FIG. 10B